Production of DNA aptamers with specificity for bacterial food pathogens

Thesis submitted in accordance with the requirements of
the University of Chester for the degree of Doctor of Philosophy
by

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September 2012
University of Chester

Declaration

The work presented in this thesis is original and has not been submitted previously in support of any qualification or course.

Signed:

Date:

Acknowledgements

I want to acknowledge Danisco A/S, Denmark and The Gladstone fellowship for funding this PhD.

I would like to acknowledge my principal supervisor Prof. Graham Bonwick for the supervision and support through the whole project. I also would like to thank my Danisco supervisor Dr Mette Ryun Drasbek who has provided great support, always found time for my questions and concerns during the practical work carried out in Denmark, but also for interactive support when I was in Chester. I would like to thank my other Danisco supervisor, Prof. Niall Young for being a great innovative support and supervisor who has shared his scientific knowledge and experience. I would like to thank the rest of my supervision team, my external advisor Prof. Christopher Smith who has helped me with the project planning and problem solving, and Dr Ian McDowall who has helped me to set up my laboratory experiments.

I would like to acknowledge all the people who have been helping me with my laboratory experiments in Chester and in Brabrand. I would not have been able to do all this without your help and support. I also like to thank all my friends and the people I have made friends with during these three years in England and in Denmark. Thank you for your support and encouragement. Special thanks for the people in 'the shed'. I have been privileged to share an office with all of you.

Viimeisenä haluaisin kiittää perhettäni kannustuksesta ja tuesta. Kiitos ymmärryksestä ratkaisuani kohtaan muuttaa Englantiin opiskelemaan ja luottamuksesta siihen, että tämä on kaiken sen arvoista.

UNIVERSITY OF CHESTER, Faculty of Applied Sciences,
Department of Biological Sciences

RIIKKA MARIA KÄRKKÄINEN: Production of DNA aptamers with specificity for bacterial food pathogens

Doctor of Philosophy thesis, 215 pages/56,864 words
Supervisors: Prof. Graham Bonwick, Prof. Christopher Smith, Dr Ian McDowall, Prof. Niall Young and Dr Mette Ryun Drasbek

DATE: 19th September 2012

Key words: Aptamers, SELEX, food-borne pathogens, pathogen detection

ABSTRACT

Aptamers are biomolecular ligands composed of nucleic acids. They can be selected to bind specifically to a range of target molecules and subsequently exploited in a fashion analogous to more traditional biomolecules such as antibodies. In this study a method for selecting new aptamers which specifically bind whole live bacterial cells is described. A non-pathogenic strain of *Escherichia coli* K12 was used to develop the method. A DNA library containing 100 bases long random nucleotide sequences was produced and the aptamer selection process was repeated nine times. An enzyme-linked technique was first used to detect bound aptamers thereafter fluorimetry and fluorescence microscopy methods were used for the detection. The aptamers were cloned and sequenced and the cloned aptamers produced with fluorescent labels. The *E. coli* K12-binding aptamers were used to demonstrate the detection of the bacterial cells in a complex food matrix, namely probiotic yogurt, by using fluorescence based detection method. The aptamer selection method with some modifications was also used to select aptamers with specificity for the food pathogens *E. coli* O157, *Listeria monocytogenes, L. innocua, S. typhimurium* and *S. enteritidis*. The aptamers against *E. coli* O157 and *S. typhimurium* were cloned and the sequences and the binding properties of these aptamers were analysed. The use of *E. coli* K12 as a target organism and the aptamer sequences presented in this study, have not previously been published in scientific literature. This is also the first report where the aptamers have been used in detection of live bacterial cells in yogurt.

UNIVERSITY OF CHESTER, Faculty of Applied Sciences,
Department of Biological Sciences

RIIKKA MARIA KÄRKKÄINEN: Ruokamyrkytysbakteerien tunnistamiseen soveltuvien DNA aptameerien tuottaminen

Väitöskirja, 215 sivua/56 864 sanaa
Ohjaajat: Prof. Graham Bonwick, Prof. Christopher Smith, Dr Ian McDowall, Prof. Niall Young ja Dr Mette Ryun Drasbek

PÄIVÄMÄÄRÄ: 19.9.2012

Avainsanat: Aptameerit, SELEX, ruokaperäiset patogeenit, patogeenien tunnistaminen

TIIVISTELMÄ

Aptameerit ovat nukleiinihapoista koostuvia biomolekyyliligandeja. Niitä voidaan valita sitoutumaan spesifisesti erilaisiin molekyyleihin, ja niiden on ajateltu voivan korvata vastaavanlaisten molekyylien kuten vasta-aineiden käyttöä. Tässä työssä aptameereja valittiin tunnistamaan eläviä bakteerisoluja. Selektiotekniikan kehittämiseen käytettiin *Escherichia coli* K12 -kantaa. Työ alkoi DNA kirjaston luomisella, minkä jälkeen aptameerit seulottiin näistä DNA-molekyyleistä toistamalla selektioprosessia yhdeksän kertaa. Aluksi aptameerien sitoutumista kohdebakteeriin todennettiin entsyymitekniikkaa käyttäen, minkä jälkeen kehitettiin fluoresointiin perustuva tekniikka. Kun aptameerien sitoutuminen kohdesoluihin oli osoitettu, aptameerit kloonattiin ja sekvensoitiin. Spesifiset aptameerimolekyylit syntetisoitiin fluoresenssileimoja käyttäen. *E. coli* K12- spesifisiä aptameerejä käytettiin bakteerien detektoimiseen ruokamatriisista. Tässä työssä esimerkkimatriisina toimi probioottinen jogurtti. Aptameereja seulottiin sitoutumaan myös ruokamyrkytysbakteereihin, kuten *E. coli* O157, *Listeria monocytogenes, L. innocua, S. typhimurium* ja *S. enteritidis. E. coli* O157 ja *S. typhimurium* -spesifiset aptameerit kloonattiin ja sekvensoitiin ja näiden patogeenispesifisten aptameerien sitoutumisominaisuudet määritettiin. *E. coli* K12 kantaa aptameerien sitoutumiskohteena, tai tässä työssä eristettyjä aptameerisekvenssejä, ei ole aikaisemmin julkaistu tieteellisessä kirjallisuudessa. Aptameerejä ei myöskään ole tiettävästi aikaisemmin käytetty elävien bakteerisolujen tunnistamiseen jogurtista.

Publications

Kärkkäinen, R.M., Ryun Drasbek, M., McDowall, I., Smith, C.J., Young, N.W.G. & Bonwick, G.A. (2011). Aptamers for safety and quality assurance in the food industry: detection of pathogens. *International Journal of Food Science and Technology*, **46**, 445–454.

Kärkkäinen, R.M., Ryun Drasbek, M., McDowall, I., Smith, C.J., Young, N.W.G. & Bonwick, G.A. (2011). Aptamers. Patent application US 61/553,501.

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Abbreviations

A	Adenine
C	Cytosine
G	Guanine
T	Thymine
N	A/T/C/G
ANOVA	Analysis of variance
AuNPs	Gold nanoparticles
BB	Binding buffer
BBf	Binding buffer (filter selection)
bFGF	Basic fibroblast growth factor
BIO	Biotin
bp	Base pair
CV	Cyclic voltammetry
ΔG	Gibbs free energy
DNA	Deoxyribonucleic acid
dsDNA	Double stranded DNA
EIS	Electrochemical impedance spectroscopy
Em	Excitation/emission maxima
F	*F*-distribution
FAM	6-carboxyfluorescein
g	Gravitational force
IFN	Interferon
IMPDH	Inosine monophosphate dehydrogenase
LB	Luria Broth
LPS	Lipopolysaccharide
µl	Micro litre
MB	Methylene blue
M	Molar (mol/L)
n	Number of observations
nm	Nano metre
nt	Nucleotide

p	*p*-value
PCR	Polymerase chain reaction
pmol	Pico mole
Px	Peroxidase
QDNA	Quencher DNA
QRM	Quartz crystal microbalance
RNA	Ribonucleic acid
rpm	Revolutions per minute
s.d	Standard deviation
SA	Streptavidin
SELEX	Systematic evolution of ligands by exponential enrichment
SPR	Surface plasmon resonance
SSCP	Single strand conformational polymorphism
ssDNA	Single stranded DNA
ssp	Subspecies
SWV	Square wave voltammetry
SWNT	Single walled nanotube
TNT	2,4,6-trinitrotoluene
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

CHAPTER 1 – Introduction

1.1. BACKGROUND

Safe and sustainable food is the goal of food producers and the food industry at large, but food is often contaminated by pathogens. Such contamination can cause various illnesses, with symptoms ranging from mildly uncomfortable to life-threatening. Therefore, detection of pathogens is important for both health and safety reasons, however the detection of food-borne pathogens is of interest not only to the food industry but also in the other areas such as the maintenance of environmental quality (Bruno, 1997) and biodefence (Bruno & Kiel, 1999). Figure 1.1 shows the most common reported incidence of common zoonotic diseases in humans in the European Union in 2009 (European Food Safety Authority, 2011). Besides the commonly reported zoonoses, there are a wide range of food poisoning micro-organisms, which can affect humans, such as *Staphylococcus aureus* (Abubakar *et al*., 2007), *Clostridium botulinum* (Jay, 2000) and several groups of viruses such as Norovirus which is one of the most important causes of non-bacterial gastroenteritis (Koopmans & Duizer, 2004).

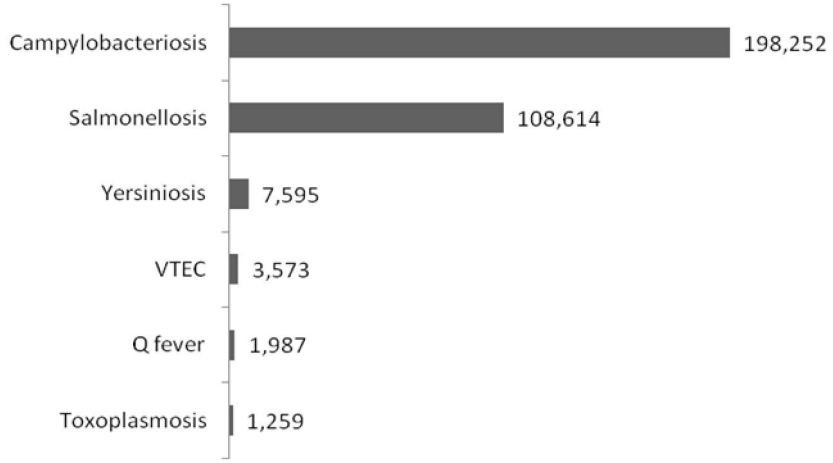


Figure 1.1 Most frequently reported zoonotic diseases in humans in the European Union in 2009 (adapted from the European Food Safety Authority, 2011).

Traditionally, culture methods have been used to detect pathogenic bacteria from food (Abubakar *et al*., 2007). The detection and identification of pathogenic bacteria relies primarily on specific microbiological and biochemical methods such as culturing in selective growth media. Conventional culture methods, although

sensitive and inexpensive, are often time and material consuming. They are typically based on the ability of the normal healthy bacterial cells to multiply in nutrient-rich medium where selective agents are often added to favour the growth of a specific group of organisms, with further biomedical tests and serological typing being used to confirm the identity of the detected pathogen (Stevens & Jaykus, 2004; Abubakar *et al*., 2007).

The number of contaminating bacteria is often low in food and water samples and initial enrichment is needed to detect pathogens. Bacteria concentration methods such as antibody-based immunocapture methods, immunomagnetic separation (IMS) (Stevens & Jaykus, 2004), filtration and centrifugation can be used. The bacterial concentration methods are not ideal because a technique optimised for one food system or micro-organism may not be applicable to other foods (Stevens & Jaykus, 2004).

Currently the principle methodologies for the rapid detection of food poisoning bacteria are well established DNA identification methods (Leonard *et al*., 2003) although antibody-based detection methods are possible (Bonwick & Smith, 2004). Using these methods has significantly reduced the detection time for pathogenic bacteria in faecal or food samples compared to traditional culture methods. *In vitro* nucleic acid amplification methods called polymerase chain reaction (PCR) (Hughes & Moody, 2007) offer several potential advantages for the rapid detection of microbial pathogens in food (Leonard *et al*., 2003; Stevens & Jaykus, 2004). The DNA sequences of different bacteria are amplified by using specific primers targeted to bacterial species or a group of different species. Variable regions within the bacterial genome, such as those found in the highly conserved 16S and 23S rRNA genes can be targeted (Bertilsson *et al.,* 2002; Hunt *et al*., 2006). The traditional PCR method is rapid and reliable but cannot be used to distinguish between dead and live bacteria cells. In some applications this is an advantage because all bacterial cells (viable and dead) need to be detected. Lazcka *et al.* (2007) reviewed different established PCR methods used for the detection of pathogens. They suggested that real-time PCR, reverse transcriptase PCR (RT-PCR) and multiplex-PCR are useful alternatives to the normal PCR method. In the PCR based techniques the enrichment

of bacterial cells is not needed even though the method could be improved if the pathogens were separated, concentrated and purified before detection.

Immunological methods, based on antibodies, are commonly used in the analysis and quality control of food materials (Bonwick & Smith, 2004) and these methods have shown to be very successful for detection of bacterial cells, spores, viruses and toxins (Iqbal *et al*., 2000). A commonly used antibody-based detection method for pathogenic bacteria is the enzyme-linked immunosorbent assay (ELISA) which makes use of either monoclonal or polyclonal antibodies (Bonwick & Smith, 2004; Karoonuthaisiri *et al*., 2009) to provide specific detection systems. The antibodies used in these assay systems are generally prepared in animals or in cell cultures derived from the tissues or organs of animals or humans. This use of animal material gives rise to a number of ethical and moral objections which makes the possibility of using an alternative 'synthesised' biomolecule attractive. Alternatives to traditional antibodies, such as single chain antibodies (Steinhauer *et al*., 2002) and peptoids (synthetic peptides) (Armand *et al*., 1998), have been investigated. Most of these alternatives have limitations in their use, such as problems of stability and low target affinity (Murphy *et al*., 2003; Phizicky *et al*., 2003).

Biosensors are devices for the detection of biological analytes. The biosensor technology is currently creating interest because it promises equally reliable results in a shorter time than traditional detection methods: PCR, colony count, and ELISA. It has been suggested that the biosensors may soon move ahead of traditional ELISA-based methods (Lazcka *et al*., 2007) and aptamers may potentially replace antibodies in biosensor applications (Mir *et al*., 2007). Aptamers often undergo significant conformational changes upon target binding and this may be one of the key factors when designing the aptamer based biosensors (Zhang *et al.*, 2008; Wang *et al.*, 2007a). It has been proposed that aptamer-based biosensors, aptasensors, can be used for the detection of pathogenic micro-organisms and viruses (Torres-Chavolla & Alocilja, 2009). Use of aptamers in biosensor applications will be further discussed in section 1.3.

1.2. APTAMERS

In 1990, Tuerk & Gold (1990) and Ellington & Szostak (1990) described short-chains, typically 15–40 nucleotides in length (Pendergrast *et al.,* 2005), of nucleotides which bound proteins with high-affinity and specificity. They called these high-affinity nucleic acid molecules, 'aptamers', a name derived from the Latin term aptus, 'to fit'. Since their discovery, the technique for isolating these single-stranded DNA or RNA aptamers has been developed (Vivekananda & Kiel, 2003; Hamula *et al*., 2008; Cao *et al.,* 2009; Kärkkäinen *et al.*, 2011a). The aptamers are capable of forming a complex with an intended target substance (Vivekananda & Kiel, 2003) and they have been used to bind to different molecular targets including proteins, bacterial cells (Hamula *et al*., 2008), viruses (Symensma *et al*., 1996), prions (Takemura *et al*., 2006), smaller molecular targets such as organic dyes (Ellington & Szostak, 1990) and nucleosides (Huizenga & Szostak, 1995).

Similar to other biomolecular ligands that exhibit binding specificity such as antibodies, aptamers have attracted interest because of their potential use as the key components of a range of analytical techniques or devices (Mir *et al*., 2006; Lee *et al*., 2007; Evtugyn *et al*., 2008; Liu *et al*., 2009). Aptamers also have a significant advantage over the other biomolecules in that they are relatively rapidly selected in vitro through SELEX (Systematic evolution of Ligands by Exponential enrichment) (Tuerk & Gold, 1990). Once they have been identified they can be inexpensively produced either synthetically or enzymatically (Pendergrast *et al*., 2005) and no animals or animal-derived cells or tissues are needed for their production. Generally aptamers are known as man-made molecules that have been isolated from synthetic random DNA or RNA libraries (Nutiu & Li, 2005) and that selectively bind small molecules, but it has been recognised that aptamer type molecules exist naturally (Winkler, *et al.* 2002; Mandal, *et al.* 2004). They are untranslated RNA messengers, called riboswitches, and they play an important regulatory role repressing or activating their cognate genes at both transcriptional and translational levels (Nudler *et al.,* 2004).

Aptamers are target specific, which means they preferentially bind to the target as opposed to other material in the wider matrix (Vivekananda & Kiel, 2003). This specificity can be an advantage when detecting pathogenic bacteria, for example in

food, if the aptamers solely bind the pathogen contrary to other micro-organisms or food matrix components. Aptamers are remarkably stable; they can be stored as a lyophilised powder at room temperature for more than one year (Pendergrast *et al*., 2005). They are also more stable at higher temperatures than antibodies, which normally function only under physiological conditions (Tombelli *et al*., 2007). The degradation of the aptamers in serum can be blocked by appropriate chemical modifications (Cummins *et al*., 1995; Dougan *et al*., 2000). These observations and the published studies (Joshi *et al*., 2009; Bruno *et al*., 2009) suggest that aptamers can also be suitable for use directly in food systems. Despite their potential, significantly more research will be necessary before they can be accepted and routinely used, particularly for the analysis of complex matrices such as food (Bruno 2009; Bruno *et al*. 2009).

1.2.1. Aptamer selection

Aptamers can be selected from a native library of single stranded (ss)DNA or ssRNA. In general, ssDNA aptamers exhibit greater stability and resistance to degradation compared to ssRNA aptamers. ssDNA is therefore likely to be a better choice for the production of aptamers for use in complex matrices such *in vivo* (Hicke *et al*., 1996) or in food (Bruno 2009). However, ssRNA might have a more variable dimensional structure than ssDNA. G-C, A-U, and G-U paring can occur in ssRNA but only G-C and A-T parings can occur in ssDNA (Pan *et al.*, 2005).

Novel aptamers can be generated through the SELEX. The process was first described by Tuerk & Gold (1990). The whole SELEX process has been reviewed by Tombelli *et al.* (2005). Briefly, the production of new DNA aptamers begins with a randomly created nucleotide sequence containing universal nucleotides flanked by 5' and 3' defined nucleotide sequences. The random region is produced by mixing equimolar amounts of each base (A, C, G, and T) and the complexity of the final library is dependent on the number of randomised nucleotides. The initial large pool of double stranded DNA (dsDNA) is produced by PCR. This dsDNA pool has to be converted into ssDNA which then forms the starter library. The principle of the PCR (Hughes & Moody, 2007) method is illustrated in Figure 1.2. The double stranded template DNA is first denatured into single stands following by a lowered temperature that allows the primers to anneal. During the extension the free

nucleotides in the solutions bind to the template forming a new complement DNA strand. These three steps are typically repeated 30-40 times to multiply exponentially the DNA template.

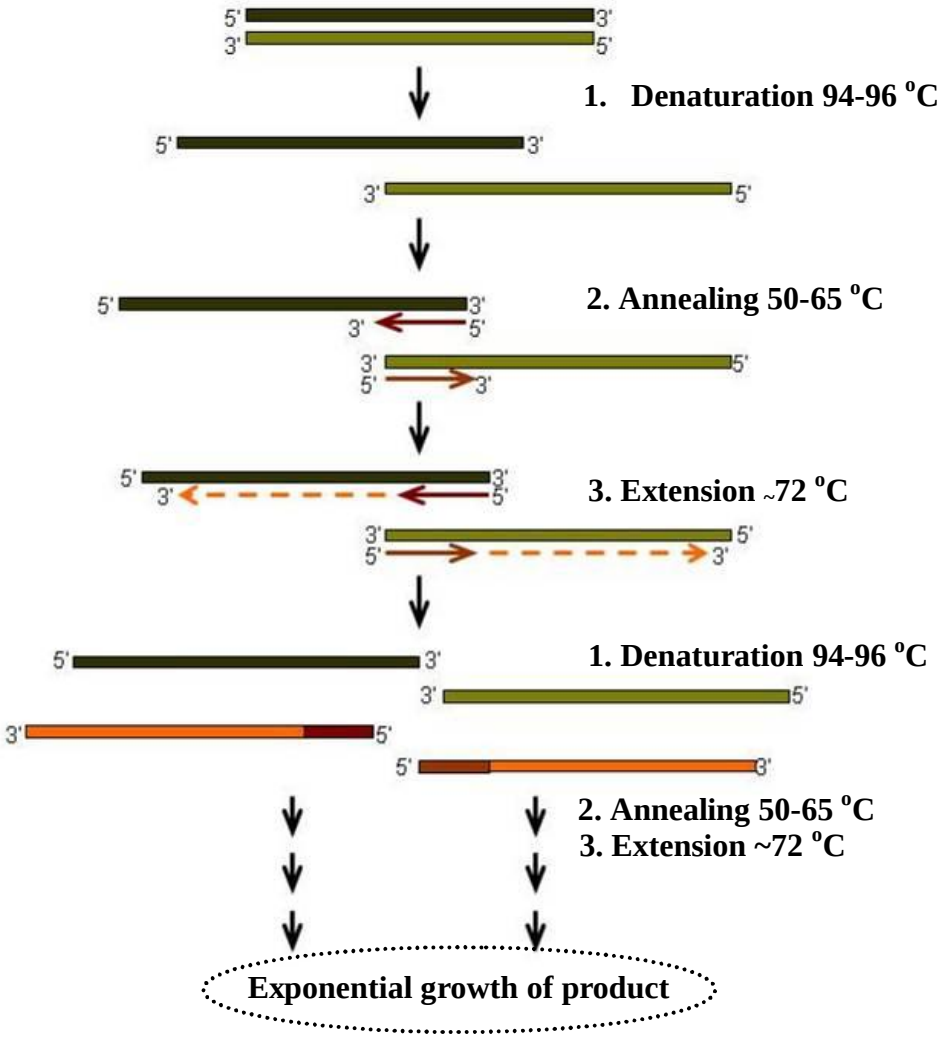


Figure 1.2 Polymerase chain reaction. 1. Denaturation separates double stranded DNA into two single strands. 2. Primers bind to the template DNA when the temperature is lowered. 3. Extension allows nucleotides to bind to the template forming a new double-stranded DNA. These three steps are repeated 30–40 times to multiply exponentially the short chain of DNA (adapted from Kärkkäinen *et al.*, 2011b).

The scheme of the aptamer selection process for DNA aptamers is illustrated in Figure 1.3. First a random DNA library has to be created. The region of the library template is produced by using the universal base (N) and the complexity of the final library is dependent on the number of randomised nucleotides. For example, a 40

bases long oligonucleotide (40mer) would consist of 4^{40} or maximally 10^{24} different nucleotide acid ligands as discussed by James (2000). During the early stages of library production and manipulation it is important not to reduce the sequence complexity much below its theoretical maximum. That allows more possibilities to find an aptamer that bind the chosen target. The isolation of the aptamers begins with the starter library of nucleic acids being incubated with the molecule of interest. The non-binding or low-binding nucleotides are separated from those aptamers that bind to the target. The separation can be done by using filtration (Vivekananda & Kiel, 2006) or centrifugation (Hamula *et al*., 2008) when the target molecules are large or by affinity chromatography (Ellington & Szostak, 1990) for small target molecules. In order to obtain only the aptamers which bind specifically to the target, negative selection using counter target molecules can be used (Cao *et al*., 2009). The aptamers which have bound the target are subsequently disassociated, eluted, collected and amplified. This process produces a less complex mixture of molecules which can be used for the next cycle of selection. Repeated rounds of selection can result in a pool of molecules with identical binding properties and possibly identical nucleotide sequences (Gening *et al*., 2001).

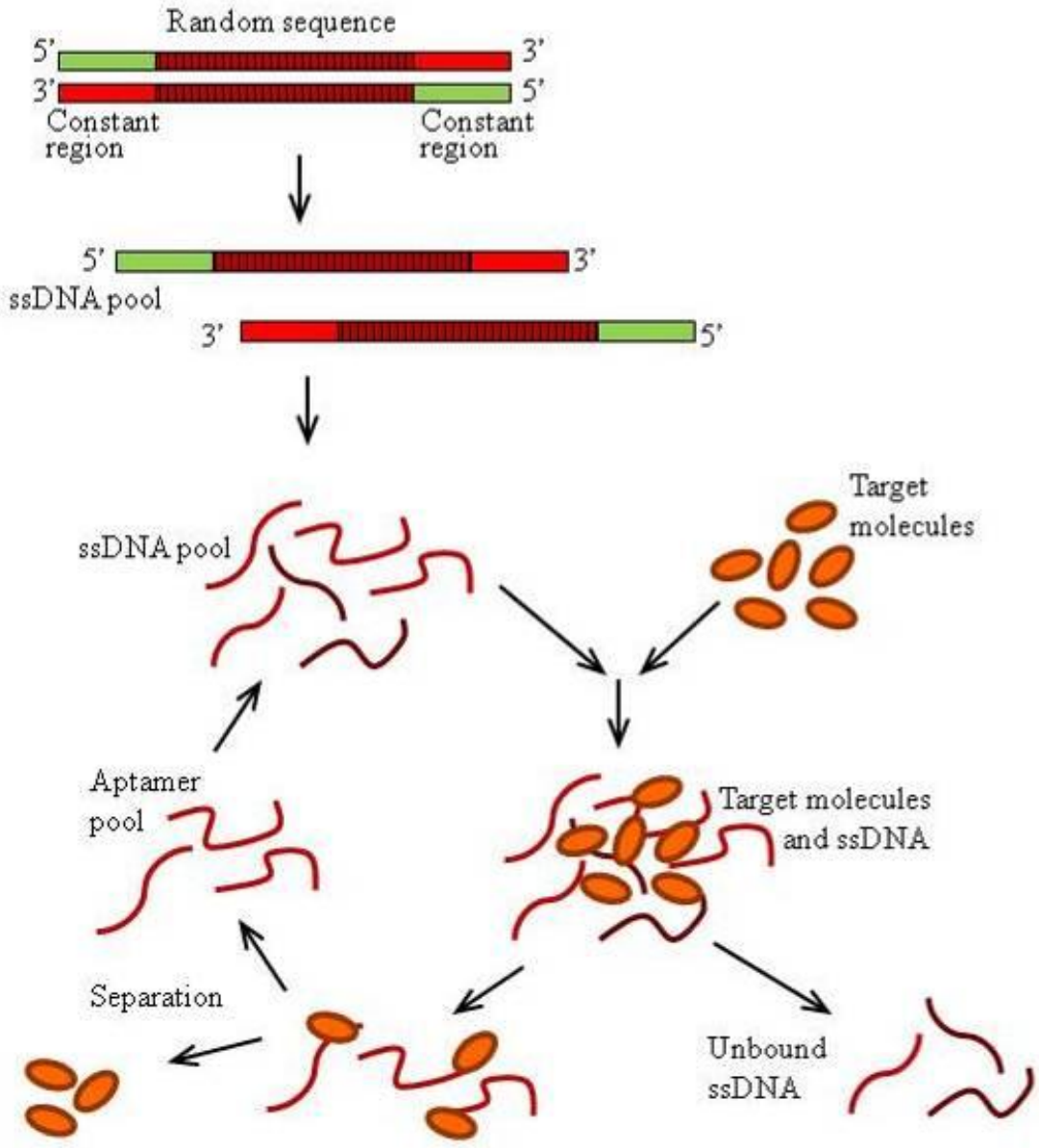


Figure 1.3 Scheme of the selection of DNA aptamers. Double stranded DNA (dsDNA) oligonucleotide library containing a randomised nucleotide sequence is synthesised and amplified by PCR. dsDNA is strand separated to single-stranded DNA (ssDNA). The target material is incubated with the ssDNA pool and the non-specific or low affinity binding nucleic acid molecules are removed. To obtain a new enriched DNA library the captured molecules has to be eluted, recovered and amplified by PCR. The whole cycle is repeated until obtaining a specific population of DNA. Finally the DNA is isolated and characterised (adapted from Kärkkäinen *et al.*, 2011b).

1.2.2. Identification of aptamer sequences

The sequence and the structure of the selected aptamers can be determined by cloning and sequencing of the nucleic acid molecules (Ellington & Stostak, 1990). The cloning and sequencing will be explained in more details in the Chapter 2 and further discussed in Chapter 5. Usually the aptamer molecules are attached to a plasmid vector that is then directed inside the competent bacterial cells. By using the normal bacterial cell cycle the vector DNA containing the aptamer insert is enriched and the aptamer sequences can then be identified. Once the sequence has been determined for an aptamer, further copies of the molecule can be produced routinely, reproducibly and in quantity by chemical synthesis based on phosphoramidite chemistry (McBride & Caruthers, 1983). This offers significant advantages over the use of antibodies in that the need for expensive and technically demanding methods of production such as cell culture may be avoided. Systematic modification, such as fluorescence dyes, amino linkers, biotin, digoxigenin and phosphorylation (Eurofins MWG Operon, 2011), of the aptamer nucleotide sequence can also be performed to produce molecules better adapted for use in diverse environments or sample matrices. Labels, such as biotin (Hamula *et al.* 2008; Vivekananda & Kiel, 2006) and fluorescent labels (Bruno *et al.* 2009; Hamula *et al.* 2008), are often added to the aptamers 5' end.

1.2.3. Advantages of labelled aptamers

Once the aptamer sequences have been identified after the SELEX, they can be synthesised. During the synthesis process, labels such as biotin, fluorescent dyes or radioactive marker can be added without inhibiting the aptamer activity. Aptamer target binding can be detected by using techniques that are based on these labels. Frequently used aptamer labels, biotin and fluorescent dyes, are introduced here with some practical examples to give an overview of the advantages of the labelled aptamers. Among the common biotin and fluorescent labels the use of radioactive (Cao *et al.,* 2009; Hesselberth *et al.,* 2000), amino-six carbon (Bruno & Kiel, 1999) and thiol labels (Cai, *et al.*, 2006; Mir *et al.,* 2006) has been reported in literature.

Biotin is a water soluble vitamin that also has a function as a carbon dioxide carrier (Campbell & Farrell, 2009). Biotin's ability to bind to streptavidin is well known (González *et al.,* 1997) and therefore it has been used as a basis for detection

platforms such as ELISA (Kittigul *et al.,* 1998). Biotin-labelled aptamers have been used in detection of aptamer binding (Mir *et al.,* 2006; Vivekananda & Kiel, 2006) but also to immobilise the aptamers on a microscope slide (Hamula *et al.,* 2008) or on magnetic beads (Joshi *et al.,* 2009). ELISA type of sandwich assay called Aptamer-Linked Immobilized Sorbent Assay (ALISA) was used by Vivekananda & Kiel (2006) to detect the aptamer binding to bacterial antigen of *Francisella tularensis.* The microtiter plates were covered with the aptamers and the antigens were then captured on the plates and biotin labelled aptamers added. Horseradish peroxidase-conjugated streptavidin was then added and allowed to bind to biotin and the colour change was indicating the amount of bound aptamers. A method based on biotin affinity to streptavidin has also been used by Bruno & Kiel (1999). The anthrax spore binding aptamers with amino-six carbon linker were captured to tosyl-activated magnetic beads. These aptamer coated beads where then used to capture the anthrax spores that was monitored with biotin-labelled reporter aptamers that were detected by an aptamer-magnetic bead-electrochemiluminiscence assay. Joshi *et al.* (2009) have shown that biotin-labelled aptamers attached to streptavidin-coated magnetic beads can be used to capture bacterial cells from real food samples. Biotin-labelled aptamers have also been used in labelling cells (Terazono *et al.,* 2010) by adding Qdot-streptavidin conjugates to biotin-labelled aptamers that have bound to the cell.

Whole spectra of fluorescence dyes are commercially available but green dyes such as 6-carboxyfluorescein (FAM) and FITC are most commonly used in the aptamer assays. Fluorescent-labels have not that variable prospects in use than biotin and has mainly been used in detection of aptamer binding. When a fluorescent-label has been attached to the aptamer the bound aptamers can be detected with different ways such as with flow cytometry (Hoffmann *et al.,* 2007; Cao *et al.,* 2009), fluorescence microscopy (Cao *et al.,* 2009; Kärkkäinen *et al.,* 2011a) and DNase footprint assay (Joshi *et al.,* 2009).

1.3. APTAMER BASED BIOSENSORS

1.3.1. Biosensors – background

Biosensors are analytical devices that are based on biological components and can be used to detect biological analytes. Biosensor applications can differentiate biological

recognition elements such as enzymes, antibodies and nucleic acids, to detect the target molecule (Lazcka *et al.*, 2007). Cheng *et al.* (2009) suggested that a typical biosensor contains three components: a biological sensing element that can recognise or bind the analyte, a transducing element which converts the detection event into a measurable signal, and a display that transforms the signal into a digital format. The sensing element primarily defines the selectivity and sensitivity of the biosensor. The detection of the analytes is usually based on sensing the analytes with either an electrical (Liss *et al.*, 2002; Tombelli *et al.*, 2005; Liu *et al.*, 2009) or optical (Baldrich *et al.*, 2004; Wang *et al.*, 2007b; Lautner *et al.*, 2010; Ohk *et al.*, 2010) readout. A problem in development of biosensors is the failure of most biomolecules to produce an easily measured signal upon target binding. For example, antibodies normally do not change their shape or dynamics when they bind to their target.

It has been suggested that the biosensors should be simple to design, easy to operate, give a fast colour change, and have minimal consumption of materials (Liu & Lu, 2006). The most commonly used biosensor is a glucose biosensor used by people suffering from diabetes mellitus to monitor their blood glucose levels (Wang, 2008). Biosensor technology is currently creating interest because it promises equally reliable results in a shorter time compared to more traditional detection methods such as PCR, colony count, and ELISA. It has been suggested that biosensors may soon move ahead of traditional ELISA-based methods (Lazcka *et al*., 2007).

Some examples of rapid biosensor platforms for the detection of bacteria will be now introduced. A highly sensitive and specific RNA biosensor for the rapid detection of viable *E. coli* in water was developed by Baeumner *et al.* (2003). This biosensor can detect as few as 40 bacterial cells in 15-20 minutes. The detection of this portable, inexpensive and very easy to use biosensor was based on the amplification of mRNA. A biosensor to detect food-borne pathogens was developed by Muhammed-Tahir & Alocilja (2003). It was a conductometric biosensor that provided a specific, sensitive, low volume, and near real-time detection mechanism for food-borne pathogens. The biosensor is based on electrochemical immunoassay which are biosensors constructed with antibodies as biological elements, attached to an electrochemical transducer. In their study the enterohemorrhagic *E. coli* O157:H7 and *Salmonella* ssp. which are of concern to biosecurity were used. It was suggested

that the method can be changed for detection of other food-borne pathogens by changing the specificity of the antibodies. The possibility of changing the antibodies used as biological elements to other biomolecular ligands such as aptamers in biosensor applications has been suggested (Mir *et al.,* 2007). This change enables a rapid method to detect pathogenic bacteria. The advantages of using aptamers over antibodies would be the lower costs of production and there are no ethical issues when aptamers are used because they can be produced by a chemical synthesis where no animals or animal cells are needed.

It has been proposed that aptamer-based biosensors, aptasensors, can be used for the detection of pathogenic micro-organisms and viruses (Torres-Chavolla & Alocilja, 2009). In general, aptamers can be a very good substitute for antibodies because they are easy to handle and they are stable compared to biologically generated proteins. Aptasensors also provide an advantage in chemical stability compared to antibody-based affinity biosensors (Liu *et al.,* 2010). Liss *et al.* (2002) demonstrated that the performance of the aptamers as immobilised ligands in biosensor application can be as good as antibodies when considering the sensitivity and specificity. Better performance was also found in terms of stability and reusability of the biosensor as the aptamer biosensor was found to be relatively heat resistant and stable over several weeks and it can tolerate repeated affine layer regeneration after ligand binding.

1.3.2. Aptasensors – Aptamer based biosensors

It has been suggested that aptamers could be used as biological recognition elements of biosensors. That an aptamer has bound to its target does not mean it can be used in a biosensor as it is necessary to have a measurable signal from a binding event between the aptamer and the target. When the aptamers bind to their target they usually undergo significant conformational changes. This has been suggested to be one of the key factors when designing the aptamer based biosensors (Wang *et al*., 2007b; Zhang *et al.,* 2008). The aptamer based biosensors often use immobilised aptamers as recognition elements for the target molecules. The most popularly used electrode material is gold where the thiolated DNA/RNA strands, in this case aptamers, can be immobilised via strong Au-S linkage (Herne *et al.,* 1997; Steel *et*

al., 1998). A streptavidin-biotin linkage has also been used (Hamula *et al.,* 2008; Joshi *et al.,* 2009).

Both, RNA and DNA aptamers have been used in biosensors. It has been established that the unmodified RNA aptamer based biosensors can be used only for a single measurement in biological media because of the degradation of the RNA by the ribonucleases (McCayley *et al.,* 2003). DNA aptamer based assays have been shown to be reusable with minimal or no change in sensitivity (Lee & Walt, 2000; Liss *et al.,* 2002; Minunni *et al.,* 2004; Liu *et al.,* 2009). These findings would suggest the DNA based aptamers are more suitable for the biosensor applications even though the stability of RNA aptamers could be improved with modification of the aptamer structure or by adding ribonuclease inhibitors.

Thrombin binding aptamers are well established and thrombin is the most commonly used analyte when developing aptamer based biosensors (Hall *et al.,* 2009; Torres-Chavolla & Alocilja, 2009). Also the first reported aptamer based biosensor was used for thrombin detection (Potyrailo *et al.,* 1998). In the study of Potyrailo *et al.* (1998) anti-thrombin aptamers were fluorescently labelled and immobilized on a glass support. The binding of thrombin to the aptamers was demonstrated by detecting the changes in the evanescent-wave-induced fluorescence anisotropy of the immobilised aptamer.

There are already a wide range of different aptamer based biosensor techniques published in the scientific literature with aptasensing platforms and aptamer detection assays having also been extensively reviewed (Fischer *et al*., 2007; De-los-Sanots-Alvarez, *et al.*, 2008; Song, *et al.*, 2008; Sassolas, *et al.*, 2009). Some of the recent aptamer based biosensors will be introduced. The aptamer biosensors are roughly divided into two different groups: the biosensors where the interaction between the aptamer and the analyte is detected by optical readout, or by electrochemical readout. There are suggestions that the electrochemical aptasensor may offer greater signal stability, sensitivity, and ease of calibration compared to fluorescence aptasensors (Liu *et al.,* 2010).

1.3.3. Optical platforms

Optical platforms use colour or fluorescence labels in detection of the aptamer binding to the analyte. Biosensors based on surface plasmon resonance (SPR) can be used for label-free analysis of biomolecular interactions, providing data on selectivity, affinity and kinetics (Näslund *et al*., 2006). SPR has been used to study the interactions between the aptamers and their targets (Baldrich *et al.* 2004; Tombelli *et al.,* 2005; Wang *et al.*, 2007b; Lautner *et al.,* 2010). This technique relies on the change of the optical parameter upon changes in the layer closest to the sensitive surface. Other platforms such as fibre optic biosensors (Lee & Walt, 2000; Ohk *et al.,* 2010), colorimetric sensors (Liu & Lu, 2006), fluorescence based biosensors, where the fluorescence signal is due to the conformation change of the aptamer (Nutiu & Li, 2003; 2004; Hall *et al.* 2009; Tuleuova *et al.,* 2010), or fluorescence polarisation (McCauley *et al.,* 2003), and gold nano-particle based biosensors (Wang *et al.* 2007b; Zhang *et al.*, 2008) have been developed.

1.3.3.1. Surface Plasmon resonance

SPR imaging has been used on several occasions to study interactions between the aptamers and their targets. Tombelli *et al.* (2005) used previously reported RNA aptamers (Yamamoto *et al.,* 2000) as a biorecognition element to develop aptasensors for the detection of human immunodeficiency virus type 1 (HIV-1) Tat protein. In their platform the aptamers were immobilised on a gold surface and SPR was used to detect the interaction between the aptamer and protein. Analytical performance (sensitivity, reproducibility and specificity) of SPR-based biosensor was studied and the immobilisation of the aptamer resulted in a very reproducible step. High selectivity was also obtained for this biosensor.

Wang *et al.* (2007b) developed an SPR biosensor for human immunoglobulin E (IgE) detection. Thrombin aptamer in Biacore platform, that uses SPR, was extensively studied and optimised by Baldrich *et al.* (2004). The thiolated trombin binding aptamers were immobilised at gold surfaces by self-assembly and the aptamer thrombin interactions were studied by SPR. They found out that different parameters, such as immobilisation strategy, incubation time and temperature, and buffer composition should be optimised for each aptamer. They also suggested that all the

aptamers are unique to its structure and a considerable study of assay parameters is necessary for the elucidation of the optimal system.

An SPR based DNA aptamer biosensor for the detection of apple stem pitting virus (ASPV) coat proteins PSA-H and MT32 was proposed by Lautner *et al.* (2010). In their aptasensor, the thiolated aptamers were immobilised onto a gold sensor chip surface and different parameters affecting this binding, such as the aptamer flanking, surface coverage, and type of spacer molecules, were identified and their influence was determined. Various concentrations of the target proteins were exposed to the sensor chip and dissociation constants indicating affinity between aptamer and protein were generated with 55 nM for MT32 aptamer and 8 nM for PSA-H aptamer. The aptasensor was shown to be specific to its target proteins and the SPR signal increased when higher amounts of virus were exposed to the sensor chip.

1.3.3.2. Fluorescence platform

Fluorescence signalling aptamers have been used in many aptasensors and different fluorescence based techniques have been extensively reviewed by Nutiu & Li (2005). The same research group developed a structure-switching signalling aptamers (Nutiu & Li, 2003; 2004). Their non-fluorescent aptamer can be turned into fluorescence-signalling reporter when the target molecule is available. In their study the aptamer binds to a DNA sequence modified with a quencher (QDNA) in the absence of the aptamer target. When the system is exposed to the target analyte the aptamer binds to the target instead of the QDNA. This conformation change means that the quencher effect of the QDNA disappears and the fluorescence signal of the aptamer can be detected.

Similar to structure-switching aptasensors, aptamer beacons (Hall *et al.*, 2009) that work in similar way to the molecular beacons (Tyagi & Kramer, 1996) has been reported. An interaction of the aptamer beacons with the analytes leads to a separation of fluorophore and quencher. Hall *et al.* (2009) generated a series of thrombin aptamer beacons. A fluorophore and quencher were included in the aptamer. An addition of thrombin leads to a conformation change of the aptamer and a separation of the fluorophore from the quencher. They developed two different thrombin aptamer beacons that had fast activation rates at 25 ^{o}C. Tuleuova *et al.*

(2010) demonstrated an aptamer beacon that emits a fluorescence signal directly upon binding to IFN-γ molecules without the need for secondary labels or washing steps. They suggested their aptasensor could be used for dynamic monitoring of cytokine release. This aptasensor suffered from problems related to stability and photobleaching of fluorophores.

An aptamer array sensor was developed for the multiplex detection of four analytes in biological matrix such as human serum or cellular extract (McCauley *et al.*, 2003). The assay, that is expected to speed up the diagnosis of cancer, was targeted to detected thrombin and the cancer associated targets inosine monophosphate dehydrogenase (IMPDH), vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The transduction principle was based on fluorescence polarization.

1.3.3.3. Colorimetric platform

The optical colorimetric signalling has been reported extensively by Liu & Lu (2006). They developed a fast colorimetric sensor based on the disassembly of nanoparticle aggregates linked by aptamers. In their study, the sensors were developed to detect adenosine and cocaine. The adenosine detecting biosensor was made of nanoparticles containing three components: gold nanoparticles functionalised with 3'-thiol-modified DNA, or 5'-thiol-modified DNA, and a linker DNA molecule. A similar gold nanoparticle-based (AuNPs) simple readout technique was developed to detect target analytes with aptamers by naked eye (Wang *et al.* 2007b). The technique was simple in design as no oligonucleotide labelling or AuNPs modification was needed. AuNPs have also been used in detection of small molecules, such as adenosine and potassium, by Zhang *et al.* (2008). Their strategy relies on the size-dependent SPR properties of AuNPs probes and aptamers.

1.3.3.4. Fibre-optic platform

A fiber-optic biosensor to detect thrombin was developed by Lee & Walt (2000). The aptamers were immobilized on the surface of silica microspheres and the binding of the protein was monitored in a microarray system. A similar fibre optic based aptasensor was developed by Ohk *et al.* (2010). Their antibody-aptamer functionalized fibre-optic biosensor can be used in the detection of *Listeria*

monocytogenes from food. The sandwich fiber-optic biosensor was based on an aptamer, specific for an invasion protein of *L. monocytogenes* together with an antibody. The antibody was immobilised on a surface to capture the target bacteria and aptamer was used as fluorescence-labelled reporter.

1.3.4. Electrochemical platforms

Aptamer based electrochemical biosensors have been extensively reviewed by several authors (Willner & Zayats, 2007; Lee *et al*., 2008; Cheng *et al*., 2009; Sassolas *et al.,* 2009). Typical, electrochemical aptasensor use an electrode surface as the platform to immobilise the aptamers. The binding of the analyte is then monitored based on electrical current variations. Detection methods, such as for example; quartz crystal microbalance (QRM) (Liss *et al.,* 2002; Tombelli *et al.,* 2005; Hianik *et al.,* 2007), electrochemical impedance spectroscopy (EIS) (Xu *et al.,* 2005; Min *et al.,* 2008; Liu *et al.,* 2009; Ho, *et al.,* 2012) and several different voltammetry methods, such as cyclic voltammetry (CV) (Cheng *et al.,* 2007; Liu *et al.*, 2009) and square wave voltammetry (SWV) (Liu *et al.,* 2009) have been used for detecting the binding events between aptamers and analytes. The sensors are normally based on the changes in aptamer configuration, conformation, and conductivity of the aptamer-containing DNA construct upon binding an analyte, respectively. Some aptasensors based on electrochemical detection are introduced here to give a short overview of the aptamer based electrochemical biosensors.

1.3.4.1. Quartz crystal microbalance

QCA was developed to detect human IgE (Liss *et al.*, 2002). Similar technique based on QCM was also used by Tombelli *et al.* (2005) to detect interactions between the HIV-1 Tat protein and a RNA aptamers developed by Yamamoto *et al.* (2000). QCM measures the frequency variation caused by adsorption of a mass of analyte at the oscillator. By using this technique, the binding event can be followed in real time. In the study of Liss *et al.* (2002), the aptamers were immobilised on a gold layer and the detection system was compared to the similar system based on antibodies. They demonstrated that aptamers were equivalent to antibodies in terms of specificity and sensitivity and their aptamer based biosensor can be reused with only a little loss of sensitivity. They have also demonstrated aptamer based biosensors to be relatively heat resistant and stable over several weeks.

1.3.4.2. Electrochemical impedance spectroscopy

EIS detection method for aptamer-modified array electrodes, where binding of the immobilised aptamers leads to impedance changes, has been developed by Xu *et al.* (2005). It was shown to be a promising label-free detection method for human IgE. A similar electrochemical reusable aptamer biosensor based on EIS was also developed by Liu *et al.* (2009) to detect small molecules. They used adenosine as a target molecule in the development of the biosensor. The modified aptamer made a structure switching when binding to its target. The aptasensor detected this structure switch by EIS and CV. The proposed techniques possessed the potential of wider application for other targets.

Simple, label-free and direct electrochemical sensor to detect interferon (IFN)-γ with aptamers was developed by Min *et al.* (2008). The aptasensor they developed was not based on the conformation change of the aptamers when interacting with the analyte, but the detection was based on the change in charge transfer resistance at the electrode interface. EIS was used to detect the interaction between the immobilised aptamer. Similar picomolar detection limits for IFN- γ was found in this study when compared to the IFN- γ aptamesensor developed by Liu *et al*. (2010). This biosensor will be introduced in section 1.3.4.4.

EIS has been recently used in several aptasensors. For example Ho *et al.* (2012) reported a biosensor for the detection of the explosives. In their sandwich biosensor the aptamers are targeted to 2,4,6-trinitotoluene (TNT). EIS is used to detect the interaction between the primary amine of TNT and anti-TNT aptamer. One of the most recent reported aptasensor that can be used in detection of bacterial cells is developed to detect lipopolysaccharide (LPS) that can be found on a surface of gram negative bacteria (Kim *et al.,* 2012). Thiolated aptamers were used to construct a biosensor where aptamer molecules function as a detection probe. The interaction of the aptamer and LPS was monitored by measuring the electrochemical impedance changes. The electrochemical aptasensor they developed showed a linear detection range from 0.01 to 1 ng/ml of LPS.

1.3.4.3. Cyclic voltammetry

Cheng *et al.* (2007) developed an aptamer based electrostatic interaction approach to detect the lysozyme enzyme that is known to damage bacterial cell walls. A DNA aptamer for lysozyme was immobilized on a gold surface by means of self-assembly and $[Ry(NH_3)_6]^{3+}$ bound to the DNA phosphate backbone via electrostatic interaction. The surface density of aptamers can be determined by measuring the $[Ry(NH_3)_6]^{3+}$ reduction peak height in the CV. Upon target binding of lysozyme to the aptamer, the surface bound $[Ry(NH_3)_6]^{3+}$ cations are released. This can be detected as a decrease in the integrated charge of the reduction peak. As mentioned previously, Liu *et al.* (2009) used CV as well as EIS as a part of their aptasensor to study interactions between aptamers and small molecules.

1.3.4.4. Square wave voltammetry

A biosensor based on the conformation change of the aptamer was developed by Liu *et al*. (2010). The electrochemical DNA aptamer-based biosensor was developed for detection of IFN-γ. The biorecognition element of the biosensor consisted of thiolated DNA hairpin carrying a redox tag. A DNA hairpin containing IFN-γ-binding aptamer was thiolated, conjugated with methylene blue (MB) redox tag, and immobilised on a gold electrode by self-assembly. The aptamer hairpin unfolded when it was bound to its target. The unfolding of the aptamer pushed MB redox molecules away from the electrode and caused a decrease in electron-transfer efficiency. SWV was used to quantify the change in redox current. The biosensor was specific to IFN-γ and showed limit of detection of 0.06 nM with linear range extending to 10 nM. The advantage of this method is that the aptasensor responds directly to the presence of cytokine without the needed for multiple labelling and washing steps. The aptamer-based electrochemical biosensor is suitable for dynamic monitoring of IFN-γ, and biosensor can also be reused multiple times because the aptamer layer is chemically stable. Overall, the electrochemical aptasensor was found to be sensitive and specific to IFN-γ, pointing to future applications in immunology, cancer research, and infectious disease monitoring.

1.3.4.5. Other electrochemical aptasensors

An aptasensor to detect protein trans-activator of transcription (Tat protein) of HIV-1 was developed by Minunni *et al.* (2004). In their study, biotinylated RNA aptamers

were immobilised on the gold electrode of the crystal that were previously coated by streptavidin. The interaction between the aptamers and the Tat protein were studied following the changes in the oscillation frequency of the crystal.

A label free electrochemical beacon type method for aptamer biosensors has been developed by using intercalators that bind to double stranded regions of the aptamer (Bang *et al.,* 2005). If these double stranded aptamer regions are close enough to the electrode, the intercalactors can serve as reporters. When aptamers are binding to their target analytes the possible conformational change may cause a release of the intercalactor producing a negative response. An example for this is when an aptamer for thrombin was immobilized on a gold electrode. MB intercalates into a double strand region and will be released upon target binding because of the conformational change of the aptamer. The MB cathodic peak current in the differential pulse voltammogram decreases with increasing thrombin concentration.

A potentiometric electrochemical aptasensor using single walled carbon nanotubes (SWNT) was developed to detect a non-pathogenic strain of *E. coli* (Zelada-Guillen *et al.,* 2009). Aptamers were modified with an amino group and bound to SWNT carboxyl groups. Electromagnetic field response was recorded indicating strong binding between aptamers and cells when the biosensor was exposed to *E. coli* bacterial cells. Specificity data of the device was generated by using alternative bacterial species. The aptasensor was found to be very sensitive, as low as 6 CFU/ml was detected when using complex matrices such as spiked apple juice and milk. The results of this study demonstrate a device that with minor modifications to the aptamer, can offer a highly sensitive, specific, and rapid method of detection for bacterial cells.

Although the development of aptamer based biosensors is proceeding apace (Freeman *et al.,* 2012; Kim *et al.* 2012), relatively few aptasensors have been developed for the direct detection of bacterial cells, particularly pathogenic bacterial cells. Offering detection methods with little or no preanalysis preparation, coupled with the potential to detect highly pathogenic organisms, aptamers are emerging as a cost effective tool for use in rapid diagnostics for food quality and assurance.

1.4. APTAMER TARGETS

Vivekananda & Kiel (2003) listed examples of possible targets for aptamers including proteins, peptides, carbohydrates, polysaccharides, glycoproteins, lipids, hormones, receptors, antigens, allergens, antibodies, substrates, metabolites, cofactors, inhibitors, drugs, and vitamins. The best targets for aptamers are large molecules, such as proteins, which offer the necessary surface area for interaction (Hamula *et al*., 2006). The binding of an aptamer to a specific target takes place exclusively by means of non-covalent binding processes, such as hydrogen bond formation, electrostatic interaction, Van der Waals interaction and hydrophobic interaction. Standard nucleic acid bond formation (Watson-Crick base pairing) where A binds to U or T and G binds to C does not take place (Vivekananda & Kiel, 2003). Similar to antibody–antigen interactions the overall 3D shape and charge of the target molecule defines the strength of the binding affinity of selected aptamers. Target molecules have to be stable and it is useful if they can be modified to enable partitioning of the aptamer and the target to facilitate selection and amplification. For example, high specificity aptamers have been selected to bind to amino acid l-arginine (Geiger *et al*., 1996), carcinogenic aromatic amines (Brockstedt *et al*., 2004), toxins (Hesselberth *et al*., 2000) and bacterial spores (Bruno & Kiel, 1999). Ions such as Zn^{2+} have been used with aptamers to get better binding properties (Kawakami *et al*., 2000). Some aptamer targets with detailed information are presented here.

1.4.1. Prions

Fatal neurodegenerative disorders such as Creutzfeldt–Jakob disease in humans and bovine spongiform encephalopathy (BSE) in cows are diseases caused by misfolded cellular prion proteins. The amino acid sequence of the normal cellular prion protein (PrPC) is the same as that of its pathogenic isoform's (PrPSc) (Prusiner, 1998). Takemura *et al*. (2006) selected DNA aptamers against recombinant human cellular prion protein. Selected aptamers bound to recombinant and mammalian PrPC but did not bind to PrPSc and other neuroproteins. This suggests that aptamers could be produced with specificity for abnormal prion proteins and could form the basis of diagnostic procedures with a range of health and safety applications.

1.4.2. Viruses

Aptamers can be selected to bind complex target structures such as viruses. Nitsche *et al*. (2007) reported the application of high-affinity DNA aptamers, which bind the Vaccinia virus. The application used for selection was a new one-step selection method called MonoLEX. They found a 64-base aptamer, which specifically bound to orthopoxviruses. This aptamer also has an ability to inhibit the *in vitro* infection of Vaccinia virus and other orthopoxviruses in a concentration- dependent manner. An example of the aptamers targeted to virus is the study by Symensma *et al.* (1996) where RNA aptamers, that can interact tightly and specifically with the HIV type 1 Rev protein, were selected. Lee *et al.* (1995) presented short RNA oligonucleotides, which blocked the binding of the HIV regulatory protein Tat. Both Rev and Tat are proteins which are necessary for HIV-1 replication. Human influenza A virus subtype H3N2 has also been detected by aptamers (Gopinath *et al.,* 2006). In the study *in vitro* selection was carried out to find specific aptamers that bind specifically only the subtype H3N2 but not other human influenza viruses. The studies of the interaction between virus and aptamers indicated that aptamers can be used to detect viruses from different matrices. These reports also suggested that aptamer-based techniques can potentially be used to detect pathogenic viruses in contaminated environmental or food matrices.

1.4.3. Protozoa

African trypanosomes are a specific class of protozoan organisms responsible for the parasitic disease sleeping sickness (Homann & Göringer, 1999; Lorger *et al*., 2003). The detection of these parasites is often done by PCR-based methods (Cox *et al.,* 2005) but different detection methods have been introduced. Kuboki *et al.* (2003) introduced a Loop-Mediated Isothermal Amplification (LAMP) method and fluorescence *in situ* hybridisation with peptide-nucleic acid probes has been reported (Radwanska *et al.,* 2002). The first three high affinity aptamers isolated to detect the African trypanosomes bound to a surface protein located within the flagellar pocket of the parasite (Homann & Göringer, 1999). Subsequently Lorger *et al.* (2003) successfully selected small, serum-stable RNAs, which bound to a surface glycoprotein of the *Trypnosoma brucei* spp. *brucei*. It is interesting to note that these reports of aptamers specific for African trypanosomes are the first aptamers for whole-cell targets (Homann & Göringer, 1999). Also the aptamer sequence, the

identification of the particular target molecule location, and the aptamer's predicted secondary structure were reported.

1.4.4. Bacteria

By targeting aptamers against some of the surface proteins of micro-organism, the growth of the bacteria can potentially be inhibited or reduced or the secretion of toxins can be prevented. Often aptamers have been selected against purified target molecules (Homann & Göringer, 1999; Lorger *et al.,* 2003; Takemura *et al*., 2006; Bruno *et al.* 2009; Joshi *et al.,* 2009; Yamamoto, *et al.,* 2010) but it has been reported that these aptamers can be used to detect bacterial cells (McMasters & Stratis-Cullum, 2006; Bruno *et al.* 2009; Cao *et al*., 2009; Joshi *et al*., 2009). There are also reports where the aptamers have directly been selected against whole bacterial cells (Chen *et al.,* 2007; Hamula *et al*., 2008; Kärkkäinen *et al.,* 2011a).

F. tularensis is a highly infectious pathogenic encapsulated bacterium causing tularaemia and it can potentially be used as a biological threat agent (Dennis *et al.,* 2001). A set of unique DNA sequences that bind with high-affinity and specificity to the *F. tularensis* subspecies japonica antigen, have been successfully isolated (Vivekananda & Kiel, 2006). Binding affinity and specificity were illustrated by use of an analogue of the antibody-based ELISA referred to as an ALISA, as well as dot blot assays. The aptamers Vivekananda & Kiel (2006) selected could be used as a screening tool for the detection and identification of tularaemia. Further, they demonstrated the ability of the same aptamers to bind to the subspecies *F. holartica* and *F. tularensis* (SCHU 4-type A), which causes the lethal form of tularaemia.

In the study of Cao *et al*. (2009) five different ssDNA aptamers were specific to different cell surface components of *S. aureus.* They suggested that targeting several sites on the whole bacterial cell simultaneously may increase the sensitivity of detection. DNA aptamers for the capture and detection of *S. enterica* serovar *typhimurium* were selected and evaluated by Joshi *et al*. (2009). Their work presents proof-of-concept that aptamers can be used as high-affinity ligands for the capture of whole bacterial cells and subsequent detection of pathogenic bacteria. In their study the outer membrane proteins were isolated and aptamers were selected to bind them. A non-immunomagnetic capture procedure was used to concentrate whole bacterial

cells from faecal samples and representative food matrixes. *S. enterica* serovar Typhimurium was a model organism to generate aptamers that can be used for capture and subsequent PCR-based detection of the organism from complex sample matrixes (Joshi *et al.*, 2009).

As mentioned previously, the aptamers have been selected to bind to whole bacterial cells using a centrifugation to separate the non-binding molecules from the high-affinity ones. In the study of Chen *et al.*, 2007 the aptamers were selected to bind to virulent *Mycobacterium tuberculosis* with high affinity and specificity. Their work is further introduced in the following section, aptamer applications in therapeutics (1.4.1). Aptamers were selected to bind to live *Lactobacillus acidophilus* bacterial cells in the study of Hamula *et al.* (2008). The putative target molecule in their study was a S-layer protein hemag1p on the surface of the *L. acidophilus* and they estimated that 164 ± 47 aptamers were bound to a target cell.

1.5. APTAMER APPLICATIONS

1.5.1. Therapeutics

The first in vitro selected single-stranded DNA aptamer specific for a protein without nucleic acid-binding properties, was a thrombin-binding aptamer (Bock *et al.*, 1992). This aptamer is a strong anticoagulant in vitro having the ability to inhibit the protein activity by blocking the appropriate receptors with a high-affinity and specificity. Its structure (Macaya *et al.*, 1993) and binding properties (Pagano *et al.*, 2008) are well studied. Mir *et al.* (2006) used three different configurations of an electrochemical aptasensor for thrombin detection. The interaction of thrombin with aptamers was detected by the quantification of p-nitroaniline produced by the thrombin's enzymatic reaction. The other method used for the detection was an enzyme labelled sandwich format. In the third method immobilised thrombin was incubated with biotin labelled aptamer. The aptamer was quantified by the electrochemical detection of the peroxidase catalysed reaction after binding of the biotin to the streptavidin-HRP (horseradish peroxidise) (Mir *et al.*, 2006). Aptamer-based assays have also been proposed for cytokines and growth factors for use in diagnostics instead of classical ELISA tests (Tombelli *et al.*, 2007). Aptamers have been targeted to growth factors such as platelet-derived growth factor (PDGF) (Green *et al.*, 1996;

Lai *et al*., 2007) and anti-VEGF. The VEGF aptamer is in use as a drug Pegaptanib (Macugen) (Eyetech Pharmaceuticals / Pfizer, reviewed by Ng *et al.*, 2006) for the treatment of age-related macular degeneration. It has been suggested that aptamers can be used as a therapeutic treatment in some bacterial infections (Pan *et al*., 2005; Chen *et al.*, 2007). Pan *et al.* (2005) found RNA aptamers that can significantly inhibit the entry of the piliated strain of *Salmonella enterica* serovar Typhi into human THP-1 cells. This type of aptamer could serve as a tool for analysis of bacteria type IVB pilus–host cell interactions and may yield information for the development of putative new drugs against *S. enteric* serovar Typhi bacterial infections. In their study they considered that two or three aptamers may, in combination, inhibit the cell invasion effectively and could be the perfect therapeutic agent. In the study of Chen *et al*. (2007), the whole bacterial SELEX strategy was used to find the high-affinity aptamers that bind virulent strains of *Mycobacterium tuberculosis* (H37Rv). The isolated aptamer called NK2 can effectively block some surface protein components of H37Rv, decrease bacterial number, and prolong survival rate in a mouse model.

1.5.2. Biological terrorist threat agents

Traditional analytical techniques have been mainly used for the detection of biological terrorist threat agents such as bacterial cells, spores, viruses and toxins (Tombelli *et al*., 2007). The detection methods and reagents are generally target-driven therefore specific and sensitive nucleic acid-based methods have been used (Iqbal *et al*., 2000). Immunological-based detection systems, which are faster and more robust, are currently widely used. Applications, where aptamers have been used to detect biological threat agents, have already been developed for detection of infectious anthrax spores from *Bacillus anthracis* (Bruno & Kiel, 1999), the potential biological attack weapon, ricin (Hesselberth *et al*., 2000), and Gram negative bacteria *F. tularensis* (Vivekananda & Kiel, 2006).

1.5.3. Food safety and quality assurance

Assays based on the use of biomolecular ligands such as antibodies have found widespread use and their replacement with aptamers as the binding molecule can be readily envisaged. The potential for rapid and consistent production of new assays is also presented through development and use of aptamers. The potential of the

aptamers in food safety and quality assurance has been widely discussed by Kärkkäinen *et al.* (2011b). One challenge in developing sensitive assays for pathogens using whole bacterial cells is that numerous proteins are present on the bacterial cell surface. It has suggested, however, that targeting several sites on the whole bacterial cell simultaneously, rather than a single molecular target, may increase the sensitivity of detection (Cao *et al.*, 2009). Selection of more than one aptamer capable of binding to these surface proteins and subsequent use of a poly-specific mixture of aptamers may provide the required detection level, particularly in comparison to those that make use of a single mono-specific aptamer. Another potential challenge in the development and application of molecular diagnostics for the detection of food-borne microbial pathogens is the complexity of the sample matrices (Torres-Chavolla & Alocilja, 2009). Matrix components present in food samples may cause interference and therefore the isolation, concentration, and purification of the microbial targets is still frequently essential to enable detection and identification. However, the ability of aptamers to function in real, complex matrices suggests that the need for these time consuming and expensive procedures may ultimately be minimised or eliminated.

It has been reported that aptamers can be used for the detection of heat-killed and live *Campylobacter jejuni* from buffers and different food matrices such as beef extract, chicken juice containing visible fat globules and milk (Bruno *et al.*, 2009; Bruno 2009). In the sandwich assays they developed the aptamers ability to adhere to plastic was used. The aptamers were adhered to the inner face of polystyrene cuvette and after addition of the target bacteria the reporter aptamers were added. The binding was detected with a magnetic bead and red quantum dot –based sandwich assay.

The other example is shown by Joshi *et al.* (2009) where the selected aptamers to *S. typhimurium* were captured to magnetic beads. These aptamer coated beads were then used to capture bacterial cells from complex matrices such as faecal and chicken rinse samples. The samples collected by the aptamer beads were then analysed by subsequent PCR analysis. Aptamers were also used to detect *L. monocytogenes* from ready-to-eat food samples by using an antibody-aptamer functionalized fibre-optic biosensor (Ohk, *et al.*, 2010). The study shows the aptamers can be used to detect

bacterial cells from food but the method cannot be used in direct detection as 18h enrichment of bacterial cells was needed. Yamamoto, *et al.,* (2010) also used *L. monocytogenes* surface proteins as a aptamer target. They showed that aptamers may be used in diagnostic and biosensor detection technologies for food, clinical, or environmental samples.

1.6. AIMS AND OBJECTIVES

Aptamer molecules can be used as the basis of simple, rapid assays or real-time monitors of food quality. Aptamer technology may help to ensure safer food and offer the prospect of the animal-free alternatives to antibody based diagnostic tests. The aim of this study is to develop DNA aptamers with diagnostic applications in the area of food safety and quality assurance using a previously described selective evolution techniques. Initial work will focus on the optimisation of the aptamer selection techniques against bacterial cells. The initial target will be non-pathogenic *Escherichia coli* K12 followed by the aptamer selection against some food contaminant strains such as *E. coli* 0157, *Salmonella* spp. and *Listeria* spp. Salmonella is one of the most common causes of food poisoning, while Listeria and *E. coli* are also harmful and cause severe illnesses but are rarer food poisoning bacteria.

Objectives:

- Design and production of DNA library (Chapter 3)
- Development of aptamer selection process (Chapter 3)
- Aptamer selection against bacterial cells
    - Non-pathogenic strain (Chapter 3)
    - Pathogenic strains (Chapter 7)
- Development of detection method for aptamer binding (Chapter 3 and 4)
- Cloning and sequencing of the aptamers (Chapter 5)
- Binding affinity and specificity of selected aptamers (Chapter 5 and 7)
- Use of aptamers in real food matrices (Chapter 6)

Aptamers are often selected against purified proteins on the surface of the bacterial cells but whole live bacterial cells can also be used. During the aptamer selection

process, the non-binding molecules have to be partitioned from the binding molecules in order to enrich the pool of binding molecules. The target molecules could be bound to a solid support or the non-binding molecules could be separated by filtration. In this study, whole live bacterial cells will be used as targets and therefore filtration or centrifugation can be used to partition the non-binding molecules from the binding ones.

Once the aptamers have been successfully selected, the binding affinity and the specificity of these aptamers will be evaluated. A suitable detection technique for the bound aptamers will be developed based on already existing techniques. The possibility of using labelled aptamer molecules will be used as a basis when developing a detection method. In this study, applications based on biotin and fluorescent-labelled aptamers will be used. The functionality of the aptamers will subsequently be tested in food matrices. Natural probiotic yogurt containing other non-aptamer target bacteria will be used as a sample matrix. Once the specific aptamers have successfully been selected against non-pathogenic bacterial strain, the same technology will be used to select the aptamers against pathogenic micro-organisms.

CHAPTER 2 – Materials and Methods

2.1. EQUIPMENT

Bio-Rad Power supply Model 1000/500
Rich-Mond Agencies Ltd., UK

Bio-Tek Synergy HT Multi-detection Microplate reader
Labtech International Ltd., UK

Eppendorf DNA LoBind tubes
Fischer Scientific, UK

Gen5™ 1.07 Data collection and Analysis Software
BioTek Instruments Inc., UK

Hermle Z 323 K Refrigerated centrifuge, fixed angle rotor (24 × 1.5mL)
VWR International Ltd., UK

Mastercycler gradient
Eppendorf, USA

MultiScreen™$_{HTS}$ Vacuum Manifold
Millipore Ireland BV

MultiScreen Filter plates with Durapore® Membrane, 0.45µm Hydrophil Low protein binding
Millipore Ireland BV

Nikon Eclipse TE2000-U Microscope System
Nikon Corporation Ltd., UK

IPLab™ 4.0 Software
Scanalytics, Inc., USA

Olympus BX41 Clinical Microscope

Olympus Corp.

Olympus U-RFL-T Burner

Olympus ColorView Soft Imaging System

CellF 2.7 Cell Imaging Software for Life Science Microscopy

Olympus soft imaging solutions GmbH

Quantity One® 4.6.3. 1-D Analysis Software

BioRad Laboratories Inc., UK

Syngene Bioimaging GeneFlash

Synoptics Ltd., UK

Techne TC-3000 Thermal cycler

Bibby Scientific Ltd., UK

UV transilluminator

BioRad Laboratories Ltd., UK

2.2. MATERIALS

2.2.1. Reagents

10% Novex® TBE Gel

Invitrogen, Life technologies Ltd., UK

ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid))

Sigma-Aldrich Co., UK

Agar No.1 Bacteriological MC2

LabM Ltd., UK

Agarose

Fisher Scientific, UK, Thermo Fisher Scientific Inc., USA

Ampicillin

Sigma-Aldrich Co., UK

Bacto™ Agar

BD Biosciences, BD Co., USA

Bovine Serum Albumin (BSA)

Invitrogen, Life technologies Ltd., UK

D-Biotin (Vitamin B7)

Fisher Scientific, UK, Thermo Fisher Scientific Inc., USA

DNA Loading Dye (6×)

Fermentas, Thermo Fisher Scientific Inc., UK

DTT (Dithiothreitol)

Promega Co., UK

EcoRI

Invitrogen, Life Technologies Ltd., UK

EDTA (Ethylenediaminetetraacetic acid)

Sigma-Aldrich Co., UK

Ethidium bromide (EtBr; 3,8-Diamino-5-ethyl-6-phenylphenanthridinium bromide)

Fluka, Sigma-Aldrich Co., UK

FAM™ (6-Carboxyfluorescein)

Applied Biosystems, Life Technologies Ltd., UK

FluoSpheres NeutrAvidin labelled microspheres, 0.2 µm Yellow-Green

Invitrogen, Life Technologies Ltd., UK

GelRed

Biotium Inc., USA

Illustra PuReTaq Ready-To-Go™ PCR Beads

GE Healthcare, Life Sciences, UK

IPTG (Isopropyl β-D-1-thiogalactopyranoside), Dioxane-Free

Promega Co., UK

LA agar (Elliker Broth)

Fluka, Sigma-Aldrich Co., UK

LIVE/DEAD® BacLight™ Bacterial viability kit

Invitrogen, Life technologies Ltd., UK

MES (4-morpholine ethanesulfonic acid)

Sigma, Sigma-Aldrich Co., UK

Microplate Black

Sterilin Ltd., Fisher Scientific, UK

Mini Sizer 50bp DNA Ladder

Norgen Biotek Corp. CA

Nutrient agar,

LabM Ltd., UK

Nutrient Broth

Oxoid, Fisher Scientific, UK

PCR 100bp Low Ladder

Sigma, Sigma-Aldrich Co., UK

PCR Sizer 100bp DNA Ladder

Norgen Biotek Corp., CA

PCR Supermix High Fidelity (HIFI)

Invitrogen, Life technologies Ltd., UK

pGEM® -T Easy Vector System

Promega Co., UK

PureLink™ Quick Plasmid Miniprep Kit,

Invitrogen, Life technologies Ltd., UK

QIAquick gel extraction kit

Qiagen, UK

QIAquick® Spin PCR product purification kit

Qiagen, UK

Spin column PCR purification kit

NBS Biologicals Ltd., UK

Streptavidin peroxidase from Streptomyces avidinii

Sigma, Sigma-Aldrich Co., UK

Tris- Borate- EDTA buffer (TBE)

Invitrogen, Life technologies Ltd., UK

triSodium Citrate

Sucrechem products Ltd., UK

Trizma Base

Sigma, Sigma-Aldrich Co., UK

Tryptic Soy (CASO) Broth

Merk, Merc & Co., Inc., USA

Tryptone

Oxoid, Fisher Scientific, UK

X-Gal

Promega Co., UK

Yeast extract

Oxoid, Fisher Scientific, UK

Yeast tRNA

Invitrogen, Life technologies Ltd., UK

Yeo Valley Organic Natural Probiotic Yogurt

YeoValley organic, UK

2.2.2. Bacterial strains

Bacillus subtilis

University of Chester, UK

Escherichia coli K12

University of Chester, UK

Escherichia coli O157 DCS 497

Danisco A/S, Denmark

Salmonella typhimurium

University of Chester, UK

Salmonella typhimurium DCS 223

Danisco A/S, Denmark

Salmonella enteritidis DCS 1152

Danisco A/S, Denmark

Staphylococcus aureus

University of Chester, UK

Lactobacillus bulgaricus

University of Chester, UK

Lactobacillus plantarum DCS 189

Danisco A/S, Denmark

Listeria innocua DCS 17

Danisco A/S, Denmark

Listeria monocytogenes DCS 489

Danisco A/S, Denmark

Listeria monocytogenes DCS 490

Danisco A/S, Denmark

JM109 Competent cells, High Efficiency

Promega Co., UK

2.2.3. Oligonucleotides

Oligonucleotides, sequencing and labelled aptamers were obtained from Eurofins MWG Operon. The synthesis is done by machines using phosphoramidite chemistry (McBride & Caruthers, 1983). The oligonucleotides and the aptamers were analysed with Oligoanalyzer 3.1 and UNAFold (IDT – Integrated DNA technologies Inc., USA).

DNA library:

5'-ACC CCT GCA GGA TCC TTT GCT GGT ACC **40×N** AGT ATC GCT AAT CAG TCT AGA GGG CCC CAG AAT-3'

Primers

Forward PR1: 5'-ACC CCT GCA GGA TCC TTT GCT GGT ACC-3'

Reverse PR2: 5'-ATT CTG GGG CCC TCT AGA CTG ATT AGC GAT ACT-3'

Biotin labelled primers

PR1BIO: 5' BIO-ACC CCT GCA GGA TCC TTT GCT GGT ACC-3'

PR2BIO: 5' BIO-ATT CTG GGG CCC TCT AGA CTG ATT AGC GAT ACT-3'

Fluorescence FAM-labelled primers

PR1FAM: 5' FAM-ACC CCT GCA GGA TCC TTT GCT GGT ACC-3'

PR2FAM: 5' FAM-ATT CTG GGG CCC TCT AGA CTG ATT AGC GAT ACT-3'

Sequencing primers:

SP6r: 5'-TAT TTA GGT GAC ACT ATA G -3'

T7: 5'-TAA TAC GAC TCA CTA TAG GG -3'

2.2.4. Buffers and solutions

All buffers were sterilised by autoclaving at 121^{o}C for 15 min.

1× Binding buffer for aptamer selection by centrifugation (BB)

50 mM Tris-Cl, pH 7.4

5 mM KCl

100 mM NaCl

1 mM $MgCl_2$

1× Binding buffer for aptamer selection by filtration (BBf)

20 mM Tris-Cl, pH 7.5

45 mM NaCl

3 mM MgCl

1 mM EDTA

1 mM DTT

The stock solutions for the binding buffers were first prepared and autoclaved before making up to a final volume.

0.05 M Citrate buffer, pH 4.0

9.61 g Citrate acid (anhydrous)

1000 mL dH_2O

Elution buffer (filter selection)

7 M Urea

100 mM MES

3 mM EDTA

1 × PBS (Tris Phosphate buffered saline), pH 7.2

8 g NaCl

0.2 g KCl

1.44 g $Na_2 HPO_4$

0.24 g $KH_2 PO_4$

1000 mL H_2O

20 × Saline Sodium Citrate buffer (SSC), pH 7.9

3 M NaCl

300 mM triSodium citrate

1 × TE-Buffer (Tris-EDTA), pH 8.0

10 mM Tris

1 mM EDTA

50 × Tris base- acetic acid- EDTA buffer (TAE)

242 g Trizma base

57.1 mL Glacial acetic acid

100 mL 0.5 M EDTA

2.2.5. Bacterial growth media

Plate Count Agar (PC-Agar)

Tryptone solution, pH 7.0

2.5 g Tryptone

1.25 g Yeast Extract

0.5 g Glucose

500 mL dH_2O

Agar solution:

6 g Agar

500 mL dH_2O

The tryptone solution pH was adjusted and the solution was mixed with the agar. The mixture was autoclaved and plated.

Luria Broth (LB)

5 g Yeast extract

10 g Tryptone

5 g NaCl

1000 mL dH_2O

For selective antibiotic containing media, filter sterilised antibiotic was added into the autoclaved solution (25 µg/mL, 50 µg/mL or 100 µg/mL).

Indicator plates (Selective LB-Agar + ampicillin, IPTG and X-Gal)

5 g Yeast extract

10 g Tryptone

5 g NaCl

1000 mL dH_2O

pH of the solution was adjusted to 7.0 and 14 g of agar was added. Sterilised ampicillin 100 µg/mL, IPTG 0.5 mM and X-Gal 80 µg/mL were added to autoclaved solution.

S.O.C Medium

2 g Tryptone

0.5 g Yeast Extract

1 mL NaCl

0.25 mL KCl

100 mL dH_2O

Sterile filtered Mg^{2+} solution and sterile filtered glucose solution in a final concentration of 20 mM. Each was added to autoclaved solution (pH 7.0).

2.3. METHODS

2.3.1. Polymerase chain reaction

PCR reaction was performed by using Ready-to-Go PCR beads with 25 pmol of reverse PR1 and forward PR2 primers and 1µl of template DNA. The amplification parameters were 5 min at 94^{o}C for initial denaturation, denaturation at 94^{o}C for 45s, annealing at 62^{o}C for 45s, and elongation at 72^{o}C for 45s unless otherwise stated. The final elongation was 7 min at 72^{o}C. Denaturation, annealing, and elongation were initially repeated for 15 or 20 cycles depending on the reaction. The PCR products were separated on agarose gel and the products were purified from the gel with a gel extraction kit or a spin column PCR purification kit by following the manufacturer's protocols.

2.3.2. Electrophoresis

The sizes of the PCR products were estimated on 2% agarose gel in 1× TAE buffer and 0.01% GelRed or 0.05% EtBr, or on polyacrylamide gel in 1× TBE buffer and post staining the gel with GelRed. PCR samples (3 µl) were mixed with 6× loading dye and topped up with the water to achieve 1× loading dye solution before the samples were applied in the wells. The agarose gels were run for 40-60 minutes with an electric field of 80V to 210V depending on the size of the gel. Polyacrylamide gels were run for 1h in an electric field of 170V. The gels were visualised by UV transillumination and the pictures were taken and analysed.

2.3.3. DNA library production

The aptamers were selected from a pool of 100 nucleotides (nt) long DNA sequences. The 100 nt sequence contained a 40 nt long random sequence and constant regions in both ends (Vivekananda & Kiel, 2006) for the primer binding: 5'-ACC CCT GCA GGA TCC TTT GCT GGT ACC- 40×N TAA GAC CCC GGG AGA TCT GAC TAA TCG CTA-3'. This initial ssDNA library (0.1 pmol and 0.5 pmol) was amplified by PCR. The PCR products were separated on agarose gel and purified directly from the gel or with the spin columns.

2.3.4. DNA precipitation

2.3.4.1. Ethanol precipitation

One tenth of 3 M Sodium acetate and three volumes of 95-100% ethanol were added before the samples (100 µl) were centrifuged for 10 min at 16,000*g* at 4°C. The supernatant was removed and the DNA pellet washed with 500 µl of 70% ethanol (stored in -20°C) and centrifuged for 10 min as described above. The supernatant was removed and the pellet air dried before the pellet was redissolved into TE-buffer (pH 8.0).

2.3.4.2. Isopropanol precipitation

Isopropanol (0.65 volumes) was added to the DNA solution (100 µl) and mixed well before the samples were centrifuged at 16,000*g* at 4°C for 15 min. The supernatant was removed and the DNA pellet washed with 500 µl of 70% ethanol (room temperature, 23°C) to remove the salt and isopropanol residues. The samples were centrifuged for 8 min at 16,000*g* and the DNA pellet was air dried before redissolved into the TE-buffer (pH 8.0).

2.3.5. Selection of aptamers specific for live bacterial cells – Filtration method

The selection of DNA aptamers against killed *F. tularensis* bacteria was described by Vivekananda & Kiel (2006). The selection method based on filtration was followed with some modification. The double stranded DNA (dsDNA) library (2.3.3) was extracted and purified from the agarose gel and heated for 3 minutes at 94°C with an equal volume (45 µl) of binding buffer (BBf) and cooled on ice to separate the strands. To exclude the filter binding single stranded DNA (ssDNA) sequences, the ssDNA samples with an equal volume of BBf (45 µl) were applied on the

MultiScreen filter plates and drawn through the filter by using a vacuum manifold. The samples were washed three times with 50 µl of BBf and the flow through samples containing non-filter binding ssDNA sequences were collected and amplified by PCR. Ready-to-go PCR beads were used with 1 µl of template DNA (non-filter binding) and 25 µM of each primer PR1 and PR2. The amplification parameters were 5 min at 94^{o}C for initial denaturation, denaturation at 94^{o}C for 1 min, annealing at 62^{o}C for 1 min, and elongation at 72^{o}C for 1 min. The final elongation was at 72^{o}C for 10 minutes. The samples were separated on agarose gel (2.3.2) and purified with the PCR product purification kit.

Four colonies of bacterial cells from the nutrient agar were suspended into PBS following a centrifugation for 10 min at 6000*g* at room temperature. The washing step was repeated twice and the bacterial pellet was resuspended into 500 µl of BBf. The non-filter binding PCR amplified pool of DNA in BBf (100 µl) was denatured as described above and mixed with the bacterial cell suspension (100 µl). The control samples without ssDNA library were performed in parallel. After a 60 min incubation at room temperature with a gentle rotation, samples were applied on a 96 well filter plate (100 µl each well) and drawn through the filter. The unbound sequences were washed three times with 50 µl of BBf. In order to elute the bound molecules, 100 µl of boiling Elution buffer was added to the samples and the aptamers were collected and precipitated with ethanol or isopropanol (2.3.4). To enrich the pool of aptamers, the samples were amplified by PCR under the conditions previously described. The selection process was repeated by using the enriched pool of aptamers in the following rounds of selection.

2.3.6. Selection of aptamers against live bacterial cells – Centrifugation method

The selection of aptamers against live bacterial cells was first described by Hamula *et al*. (2008). In this study their protocol was followed with some modifications. Fresh overnight cultures of bacterial cells were used in every round of selection. Cell suspension (1 ml) was washed three times by centrifuging at 3500*g* for 5 min at 4^{o}C and resuspended in 500 µl of 1× binding buffer (BB). The dsDNA was denatured to ssDNA by heating at 94^{o}C for 5 minutes and then cooling on ice for 10 minutes. 100 µl of cell suspension and 25 µl of ssDNA library (PCR product) were mixed with BB containing 125 µg/ml tRNA and 0.005% BSA in order to reduce non-specific binding. Low DNA binding (LoBind) tubes were used to reduce the aptamers to bind

the tube. The mixture was incubated at room temperature with gentle rotation for 45 min. Unbound aptamers were washed three times after each of the first seven incubations and five times on the eighth round of selection with 250 µl of BB containing 0.05% BSA by centrifuging the cells at 4000*g* for 5 min at 4^{o}C and collecting the supernatant. Bacterial cells and DNA were separated and the aptamers-containing supernatant was collected. Tubes were replaced with new fresh tubes after the first and third washes in order to eliminate aptamers which may have bound non-specifically to the tube wall. After the washes the bacterial cells with bound aptamers were resuspended in 10 mM Tris-Cl (pH 8.5) and the cells were heated to 94^{o}C for 10 min to release the captured aptamers from the cells. The cells were centrifuged and the aptamers containing supernatant collected. The aptamer pool (supernatant) (1 µl) was amplified by PCR (2.3.1). The primers and other PCR parameters were the same as those used to produce the DNA library and 20 cycles of PCR amplification was repeated.

Counter selection was performed in order to eliminate aptamers that bind bacteria other than the one of interest. The counter selection was performed after selection round 5 and round 8. The protocol for counter selection was the same as that used when selecting specific aptamers except that the unbound DNA was collected and used as a new pool of aptamers. The number of PCR cycles was reduced to 15 cycles and the template concentration had to be lowered in order to obtain 100bp PCR products. The template was diluted 1:30 and 1:40.

2.3.7. Labelled aptamers

Aptamers were labelled with biotin (BIO) or 6-carboxyfluorescein fluorescence (FAM) labels by amplifying the aptamer pools by PCR (2.3.1) using 5' biotinylated or 5' FAM-labelled primers PR1 and PR2. All PCR amplification conditions remained the same. The PCR products were separated and visualised on agarose gel and the products were purified with the spin columns as described. Before the binding reaction, aptamers were strand separated by heating the aptamers at 94^{o}C for 10 min and cooling on ice immediately.

2.3.8. Detection of aptamer binding by enzyme linked technique

Fresh overnight bacterial culture was prepared as previously described (2.3.6) and biotin-labelled aptamers were produced (2.3.7). 100 µl of single stranded aptamer solution was added to an equal volume of bacterial suspension in BB in triplicate and incubated for 45 min at room temperature with gentle rotation. Unbound aptamers were washed three times by centrifuging the cells at 3500*g* at 4°C for 5 min and resuspending in BB containing 0.05% of BSA. New fresh tubes were changed via resuspension after the incubation. The cells were resuspended in the PBS containing 0.1% BSA and 1µg/ml peroxidase labelled streptavidin and the samples were incubated for 45 min at room temperature in gentle rotation to allow streptavidin to bind to biotin. Unbound streptavidin was washed three times with PBS and fresh clean microcentrifuge tubes were changed after the first and third wash via resuspension. The ABTS substrate (1%) in 0.05M citrate buffer in presence of 0.3% H_2O_2 was added and after 40 min incubation the cells were centrifuged and the absorbance of the reaction mixture measured at 405 nm. Five different dilutions of aptamers (1:2, 1:4, 1:10, 1:20, 1:40) were tested in triplicate. The reaction is illustrated in Figure 2.1.

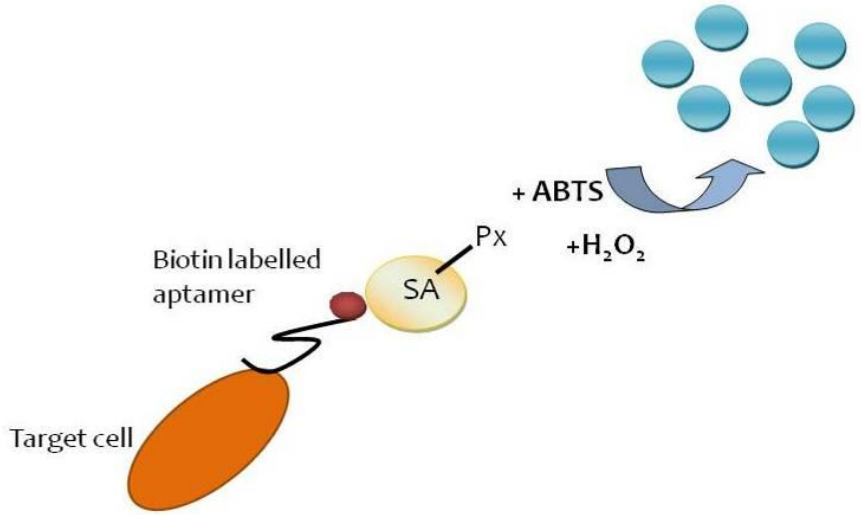


Figure 2.1 Illustration of enzyme linked technique for detection of bound aptamers. Biotin labelled aptamer bound to target cell wall and peroxidase (Px) labelled streptavidin (SA) has bound to biotin. The colour change appears when ABTS substrate reacts with peroxidase.

2.3.9. Detection of fluorescence aptamers

2.3.9.1. FAM-labelled aptamer pools binding to the bacterial cell surface

FAM-labelled aptamers were produced (2.3.7) and strand separated by heating the aptamer pool at 94^{o}C for 10 min and cooling immediately on ice. A bacterial suspension was prepared by centrifuging of fresh overnight grown culture (1.5 ml) at 3500g for 5 min at 4^{o}C. The bacterial pellet was then washed and resuspended in 500 µl of BB. The bacterial suspension (100 µl) was incubated with denatured single-stranded aptamers in triplicate if not mentioned otherwise for 45 min at room temperature in gentle rotation in LoBind tubes followed by centrifugation of 3500*g* for 5 min at 4^{o}C. Unbound aptamers were washed three times with 250 µl BB to remove the unbound aptamers. Incubation time was optimised to 45 min.

2.3.9.2. Fluorescence microscope

On a microscope slide 6 µl of bacterial cell suspension was added and the samples were viewed under the 60× (Nikon) or 100× (Olympus) magnification with the filter settings for green light (Excitation/emission maxima 520/495 nm) and normal visible light. The pictures were taken and analysed.

2.3.9.3. Fluorimetry

A fluorescence plate reader (495 nm, Em 520) was used to measure the fluorescence of the FAM-labelled aptamers with a sensitivity of 50. Washed bacterial cells with bound aptamers were resuspended in 100 µl of BB and the samples were applied to black 96-well plates. The values were compared by one way analysis of variance (ANOVA) where significant difference was $P \leq 0.05$, highly significant difference was $P < 0.01$ and very highly significant difference was $P < 0.001$.

2.3.10. FluoSpheres® Fluorescence microspheres

The bacterial suspension and the aptamer binding reaction were performed as described for FAM-labelled aptamers (2.3.10.1), except biotin-labelled aptamers were used. Biotin-labelled aptamers were produced (2.3.7) and the aptamers were strand separated by heating at 94^{o}C for 10 min and cooling immediately on ice. The bacterial cells with biotin labelled aptamers on the surface were washed once with BB and resuspended into PBS with 1% microspheres. The samples were incubated at room temperature for 45 min with a gentle rotation to let the fluoSpheres to bind to

the biotin labelled aptamers. 1% BSA was used to block the non-specific binding sites. After the incubation, samples were washed twice with PBS and the pellet was resuspended into 30 µl PBS. 6 µl of this suspension was placed on a microscope slide and the samples were viewed under 100× magnification with green light (Excitation 488 nm) and normal visible light. The pictures were taken and analysed.

2.3.11. Live/Dead® *Bac*Light™ staining

Bacterial cells were stained with Live/Dead *Bac*Light kit to distinguish the aptamer binding between the live and dead bacterial cells. The staining is based on CYTO-9 green-fluorescent nucleic acid stain that stains all the bacteria and the Propidium iodide red-fluorescent nucleic acid stain that only stains the bacteria with damaged membranes. FluoSpheres (2.3.10.2) were used to visualise the aptamer binding. Bacterial cells with biotin aptamers and FluoSpheres bound to them were resuspended into 0.85 % NaCl solution. An equal volume of staining solution (1 volume CYTO-9, 4 volumes Propidium iodide) was added to the suspension and incubated for 15 min at room temperature. 6 µl of bacterial cell suspension was added on a microscope slide and the samples were viewed under the 100× magnification with the filter settings for green (Excitation/emission maxima 480/500 nm) and for red fluorescence (Excitation/emission maxima 490/635 nm). The pictures were taken with the camera attached to the microscope.

2.3.12. Identification of aptamer sequences

2.3.12.1.Cloning of aptamers

The pGEM-T Easy Vector system was used to clone the aptamer pools. The cloning was performed by following the instructions manual (Promega Technical Manual, 2009). In Figure 2.2 is a schematic presentation of the main cloning steps. First the aptamers are PCR amplified (1. PCR) and the products purified with the spin columns. Purified aptamers are ligated to pGEM-T Easy vector by incubating in a rapid ligation buffer for an hour at room temperature (2. Ligation). The linearised vector (Figure 2.3) is designed with a single 3' -terminal thymidine (T) overhang at both ends. This improves the efficiency of ligation of PCR products by preventing recircularisation of the vector and providing a compatible overhang for PCR products generated by *Taq*-polymerases. The insert vectors are then transformed into the competent cells (Figure 2.2, 3.Transformation) and the cells are then incubated in

order to enrich the amount of the bacterial cells that have the insert vector inside (4. Cloned culture).

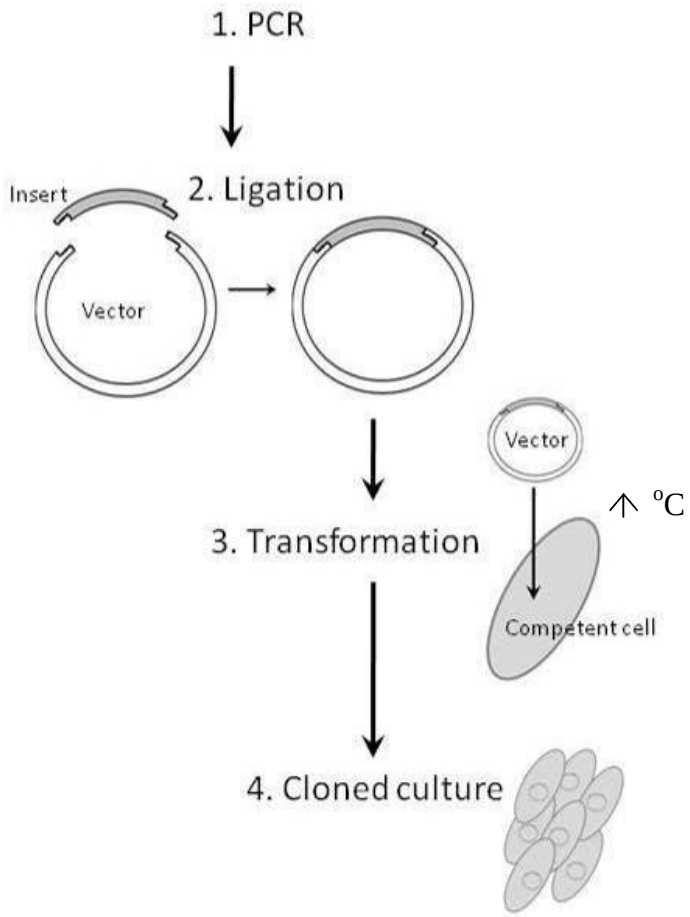


Figure 2.2 Aptamer cloning. 1. PCR amplification of the aptamer pools. 2. Ligation of the aptamers (insert) into linearised plasmid vector. 3. Transformation of the vector with an aptamer insert into the competent bacterial cells. 4. Growth of bacteria and enrichment of the cloned plasmid during the normal bacterial growth.

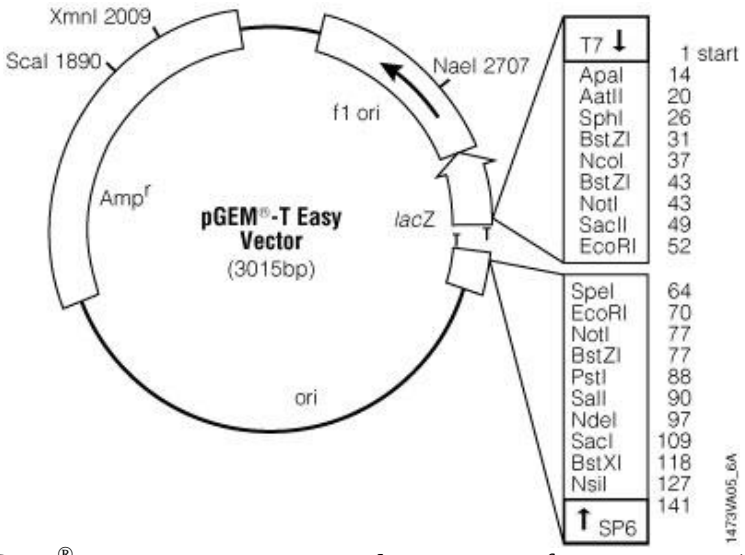


Figure 2.3 pGEM®-T Easy Vector map and sequence reference points (Promega Technical manual, 2009).

The aptamer pools 9 were PCR amplified (2.3.1) before they were ligated into the vector. Reaction components and amounts for the ligation reaction are presented in Table 2.1. Positive control with control insert DNA and background control without an insert were performed. The ligation reactions (vectors with the inserts) were first incubated on ice with the competent cells (thawed on ice for 5 min) for 20 minutes before transformed into the JM109 competent cells.

Table 2.1 Reaction components for ligation.

Reaction component	Standard reaction	Background	Positive control
Ligation buffer	5 µl	5µl	5µl
pGEM-T Easy vector	1µl	1µl	1µl
PCR product	X µl	-	-
Control insert DNA	-	2 µl	-
Ligase	1µl	1µl	1µl
H_2O to a final volume of	10 µl	10 µl	10 µl

The transformation of the vectors into the bacterial cells was done by heating the samples for 50 sec at 42^{o}C and cooled on ice for 2 minutes. Transformation efficiency was estimated by using the uncut plasmid (0.1 ng). The competent cells were then incubated in SOC medium (950 µl) for 1.5 h at 37^{o}C with 150 rpm rotation

followed by the plating of the samples (100 µl) on the selective indicator plates. The plates were incubated overnight at 37°C.

2.3.12.2. Analysing the positive colonies – Colour selection

pGEM-T Easy vector has a multiple cloning region (Figure 2.4) within the α-peptide coding region of the enzyme β-galactosidase (Promega Technical manual, 2009). The DNA insert deactivates the α-peptide and makes the colour screening of the recombinants possible on the indicator plates. Positive colonies were white on the plates and negative colonies were blue. Positive colonies were selected and transferred to a new selective plate. After an overnight incubation the clones were analysed by PCR and restriction analysis.

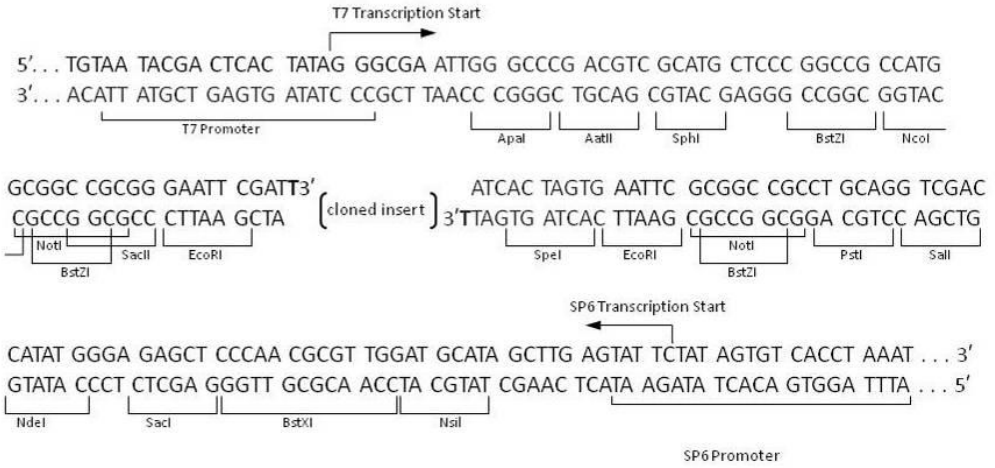


Figure 2.4 Sequence and multi-cloning site of pGEM®-T Easy Vector (adapted from Promega Technical manual, 2009).

2.3.12.3. Analysing the positive colonies – PCR analysis

For PCR analysis, PCR Supermix HiFi was used with 25 µM of both primers (PR1 and PR2) and one colony on the plate was used as a template. The amplification parameters were 10 min at 94°C for initial denaturation, denaturation at 94°C for 45s, annealing at 62°C for 45s, and elongation at 72°C for 45s. The final elongation was 10 min at 72°C. The PCR cycles denaturation, annealing, and elongation were repeated 20 times. PCR products were separated on agarose gel and the pictures were taken (2.3.2).

2.3.12.4. Plasmid extraction

One positive colony from the indicator plate was incubated overnight in 5 ml LB-broth (ampicillin 50 µg/ml). The plasmid vector was extracted from a 3 ml of overnight culture by using a plasmid extraction kit. The pure plasmid was then used for restriction analysis and sequencing.

2.3.12.5. Analysing the positive colonies- Restriction analysis

EcoRI was used as a restriction enzyme to digest the insert from the plasmid. The restriction map for pGEM-T Easy vector is shown in Figure 2.3. Enzyme EcoRI (1 µl) was added to 14 µl of sterile water with 2 µl 10× Buffer (provided with the enzyme) and 3 µl purified plasmid. The reaction was incubated at 37^{o}C water bath for 1 h. The restriction products (9 µl each) were separated on an agarose gel (2.3.2).

2.3.12.6. Sequencing of cloned vector

The aptamers were cloned by using pGEM-T Easy Vector system and the plasmid DNA was purified with Quick Plasmid Miniprep Kit. The plasmid DNA (30 µl) samples were sent in a microcentrifuge tube to sequencing. The sequencing primers (T7 and SP6r) were designed to match to vector's multiple cloning sites. The primer binding sites are marked in Figure 2.5 in red. The first forward sequencing primer (T7) was selected from the sequencing suppliers Services à la Carte (list of standard primers). The reverse primer (SP6r) was synthesised for sequencing.

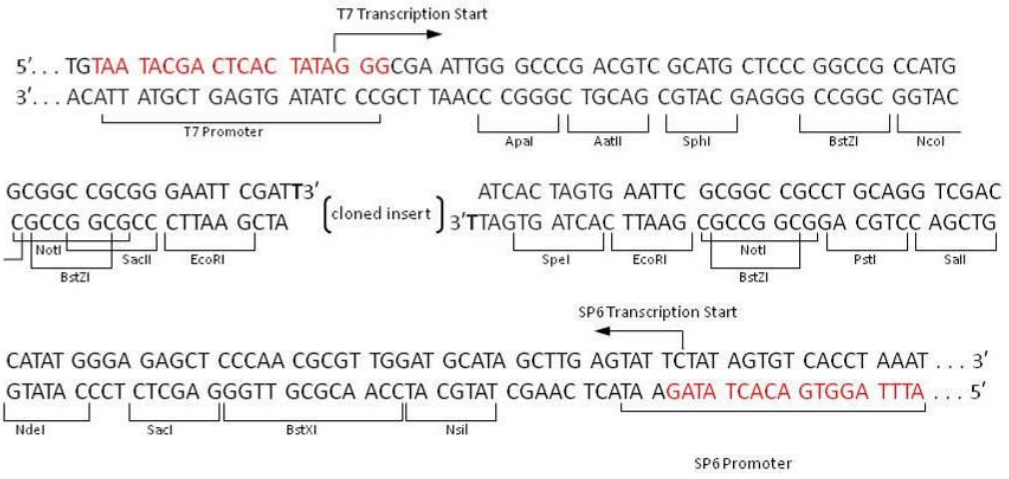


Figure 2.5 Sequence and multi-cloning site of pGEM®-T Easy Vector (adapted from Promega Technical manual, 2009) with sequencing primer sites (in red).

2.3.12.7. Aptamer sequence analysis

The 100 nt long aptamer sequences were identified from the vector sequence by comparing the primers PR1 and PR2 sequences to the vector sequence. This 100 nt aptamer sequence was analysed by UNAFold program and the length of the aptamers was reduced to 35-70 nt. Aptamers were synthesised with a fluorescence FAM-label and the binding was tested (2.3.9).

The aptamer binding to its target is dependent on its secondary structure. The possible secondary structures of the aptamer sequences were analysed using OligoAnalyzer 3.1 UNAFold program. The sequence was given to the program for analysis of sodium concentration being 100 mM and magnesium concentration 1 mM as in binding buffer (BB). The temperature was set to 25^{o}C. The most common secondary structure given was used as a template to reduce the 100 nt nucleotide sequence of the aptamers to 35 to 70 nt aptamers. The analysis of these aptamers was done with the UNAFold.

CHAPTER 3 – Selection of specific aptamers against non-pathogenic *Escherichia coli* K12

3.1. INTRODUCTION

Aptamers are single-stranded DNA or RNA ligands that can be selected to bind to proteins but also smaller molecules such as organic dyes (Ellington & Szostak, 1990) as well as prions (Iqbal *et al*., 2000), bacterial cells (Hamula *et al*., 2008) and viruses (Symensma *et al.,* 1996). Binding of the aptamers to their target mainly encompasses all types of non-covalent binding except normal standard nucleic acid bond formation (Watson- Crick base pairing). This work was focused on optimising the techniques necessary for the routine preparation of aptamers against live bacterial cells. Aptamers were selected from a randomly created DNA library using a selective evolution technique against live bacterial cells of non-pathogenic *Escherichia coli* K12. The development of selection techniques began with a technique based on filtering. Filter membranes were used to separate the non-target binding molecules from the ones binding the target. Due to the difficulties of the aptamer elution procedure of the filtering method, a new technique, that is easier and faster to perform, was introduced. The new technique, centrifugation method, is based on different weights and sizes of the molecules. Nine rounds of selection were performed and counter selection was used to deselect aptamers that were binding to bacterial cells other than *E. coli* K12. The counter bacteria used were *Lactobacillus bulgaricus* after selection round 5 and *Bacillus subtilis* and *S. typhimurium* after selection round 8.

Aptamer pools are produced by PCR and the pools can be labelled by using primers that contain labels. Biotin is one of the well reported aptamer labels (Bruno & Kiel, 1999; Mir *et al.,* 2006; Vivekananda & Kiel, 2006; Murphy *et al.,* 2003; Terazono *et al.,* 2010). In this study the aptamer pool 9 was biotin labelled and the binding of the aptamers was tested with an enzyme linked technique.

3.2. METHODS

3.2.1. DNA library production

A random 100 nt DNA library was produced as described (2.3.3). The PCR cycle (denaturation-annealing-elongation) was initially repeated over 30 cycles with 1 min reaction times resulting in the formation of non-specific products. Reaction times, temperatures, template concentration and the number of PCR cycles were optimised. Primers were analysed with an Oligoanalyzer to see the primer-dimers that can possibly be formed and their ΔG values. The PCR products were separated on agarose gel (2.3.2) and the pictures were taken. The DNA library was purified from gels with a gel extraction kit.

3.2.2. Selection of *E. coli* K12 specific aptamers

The aptamers were selected from a random DNA library to bind specifically to non-pathogenic strain *E. coli* K12. Filtration selection (2.3.5) was first used to select the aptamers but was found to be overtly complicated. The centrifugation method (2.3.6), which was easier and faster to perform, was developed and used for aptamer selection. A flow chart of the selection steps is presented in Figure 3.1.

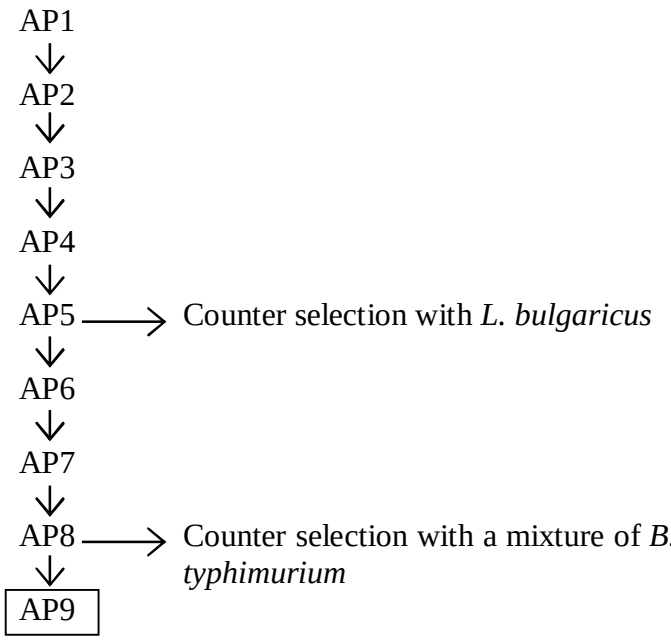


Figure 3.1 Aptamer selection steps AP1-AP9. The counter selection was performed after the fifth and eighth round of selection.

The first 5 aptamer pools were selected (2.3.6) and the pools were amplified by PCR (2.3.1) using 20 reaction cycles. The first counter selection was performed after the fifth round of selection where *L. bulgaricus* was used as a counter bacterium (Figure 3.1). The samples were collected and amplified by PCR. After the counter selection the PCR was optimised. The template was diluted to 1:30 and the number of amplification cycles was reduced to 15 cycles. The template control was performed to see if the addition of a template to the reaction appears as a band on an agarose gel.

Aptamer pool 5, that has gone through the counter selection, was used as an aptamer pool for selection round 6. Samples were amplified by PCR with 15 PCR cycles. Selection round 7 was performed as normal and both replicates were amplified by PCR twice in two separate tubes due the low PCR product yield after the previous selection round. Those two PCR products were extracted and mixed together and aptamer pool 8 was selected from this pool of aptamers in two replicates. The number of PCR cycles was increased to 20 cycles as 15 cycles did not result in a PCR product that could have be seen on an agarose gel. This increase of the PCR cycles resulted in a higher number of aptamer copies and therefore the PCR product can be visualised on an agarose gel. Two replicates were amplified in two separate reaction tubes and mixed together.

The second counter selection was done by selecting the aptamers that are not binding to a mixture of *B. subtilis* or *S. typhimurium* (Figure 3.1). Aptamer pool 8 was used for the selection and the non-binding samples were collected and amplified by PCR. The template for the PCR was diluted 1:40 and the samples were amplified for 15 cycles.

The ninth pool of aptamers was selected from the pool 8. Pool 9 was PCR amplified and separated on polyacrylamide gel (2.3.2). Polyacrylamide gel was used for these samples in order to see which one, agarose or polyacryamide gel, is more suitable for separating the aptamer PCR products.

3.2.3. Biotin labelled *E. coli* K12 binding aptamers

Aptamer pool 9 specific for *E. coli* K12 was biotin-labelled by PCR using 5' biotin-labelled primers (2.3.7).

3.2.4. Detection of *E. coli* K12 binding aptamers by enzyme linked technique

The biotin-labelled aptamer pool 9 selected to bind *E. coli* K12 was incubated with bacterial culture (2.3.8). Bacterial cells with biotin-labelled aptamers bound to them were incubated with streptavidin peroxidase following the addition of ABTS and H_2O_2. The colour change was detected by measuring the absorbance of the samples at 405 nm. Five different dilutions of aptamers (1:2, 1:4, 1:10, 1:20, 1:40) were tested in triplicate. The results were analysed with the analysis of variance (ANOVA).

3.3. RESULTS AND DISCUSSION

3.3.1. DNA library production

In this study ssDNA aptamers were selected because DNA is more stable and not that easily affected by nucleases than RNA. Also the selection of ssDNA aptamers requires one less step because there is no need for the transcription of the DNA to RNA. Even the ssDNA may be more stable in complex matrices as in food ssRNA might have more variable dimensional structures (Pan *et al.,* 2005).

The random DNA library was produced by PCR and the samples were separated on agarose gel. When the PCR amplification reaction was repeated for 30 cycles, and the reaction (denaturation, annealing and elongation) times were 1 min, non-specific products were observed. In these conditions non-specific PCR products were appearing on agarose gel pictures as extra bands (Figure 3.2). In lane 0 is a PCR control, where only PCR primers PR1 and PR2 are added, products smaller than 100 bp and a faint 100 bp band can be seen. In lane 1 where the template DNA is added, two bands can unexpectedly be seen. These extra bands can be caused by the dimerisation of the primers, for example, formation of self-dimers or hetero-dimers. In figure 3.3 the strongest primer-dimers likely to be formed with the primers PR1 and PR2 are presented. Nevertheless, the strongest homo-dimer (Oligoanalyzer) having a ΔG -16.38 kcal/mol is not as strong as the bonding between the primers and their complementary target sequences (ΔG for PR1 -55.47 kcal/mol and for PR2 -

62.09 kcal/mol. It has also been demonstrated that over amplification can lead to a loss of the complete product (Musheev & Krylov, 2006). Also PCR can yield undesired products due to properties of different (random) DNA template sequences (Viswanathan *et al.*, 1999). Kang *et al.* (2005) suggested that the heterogeneity of template sequences with different GC contents can cause non-uniform amplification during the PCR because of secondary structure formation. For example, GC rich regions can cause the formation of loop structures, which, in turn, causes polymerase jumping and production of shortened products (Musheev & Krylov, 2006). It has been shown that the heterogeneous nature of the library leads to rapid product conversion to by-products (Musheev & Krylov, 2006). The dynamics of the product and the by-product formation depends on the design of the library and the primers. It was suggested that it is not dependent on the initial concentration of the library but on the concentration of the DNA polymerase. Therefore the PCR amplification of random DNA libraries should be carefully optimised for efficient aptamer selection in SELEX. In this study PCR beads were used and therefore the concentration of the DNA polymerase was not optimised but it was standard (0.4 U/µl) in all PCR reactions.

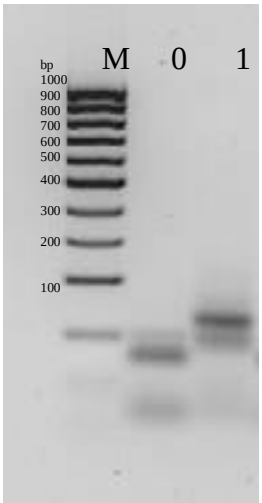


Figure 3.2 2% Agarose gel with the DNA library and non-specific products. Lane M on the gel contains PCR Sizer 100bp DNA Ladder, lane 0 is a PCR control and in lane 1 is a PCR amplified DNA library with 2.5 pmol template DNA.

Figure 3.3 Homo- and heterodimers that can be formed between the primers PR1 and PR2. The oligonucleotides were analysed with an Oligoanalyzer.

PCR products without non-specific products were achieved when the number of the PCR cycles was reduced to 15 or 20 and the reaction times were reduced from 1 min to 45 s. PCR control sample appears to be clear and non-specific bands cannot be observed on gel pictures. The DNA library was produced by using these conditions and the agarose gel of the library is shown in Figure 3.4. The samples (DNA library) are on gel in lanes 1-6 and can be seen as thick bands. Two different template concentrations were used in PCR reaction. In lanes 1, 2 and 3, 0.1 pmol template was added and in lanes 4, 5 and 6, 0.5 pmol template was added. It can be seen that this change in concentration did not affect to the actual PCR yield. In lane 0 is the PCR control sample where no template DNA was added. The faint band, smaller than 50 bp, is the primer dimer. The PCR products were expected to be 100 bp in size because the DNA library size was 100 nucleotides. The bands on the gel seem to be 140 bp instead of 100 bp. It has been found that the pre-stained agarose gels might affect the mobility of DNA on gel and especially small DNA fragments might be affected (Miller *et al*. 1999) making it difficult to estimate the size of the PCR product on agarose gel. It is also expected, when the PCR yield is large, that the

samples do not move on the gel as fast as the samples with containing less DNA. Therefore the actual product size in this study is very likely to be 100 bp. This will later be confirmed when the aptamers were cloned. Although, it has been demonstrated that the longer PCR products have been formed when the aptamers have been produced (Bruno, 1997). This may be due to partially hybridised random regions that concatenate to form a longer product or triple helical DNA formation.

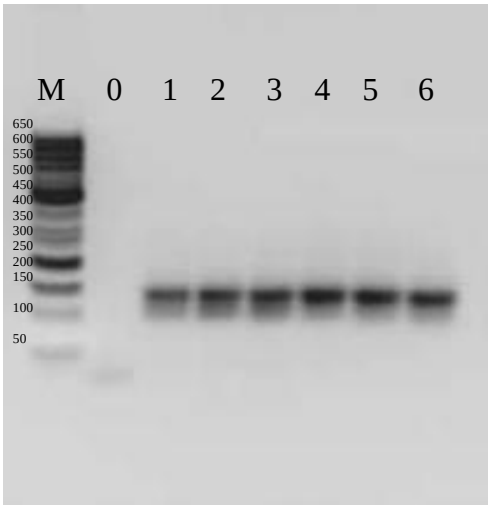


Figure 3.4 2% Agarose gel with DNA library. Lane M on the gel contains PCR MiniSizer 50bp DNA Ladder, lane 0 is a PCR control sample (no template DNA added), lanes 1, 2, and 3 are DNA library with 0.1 pmol template DNA and lanes 4, 5, and 6 are DNA library with 0.5 pmol template DNA.

3.3.2. Selection of *E. coli* K12 specific aptamers

The selection of the aptamers has been explained and illustrated (1.2.1). In this study, aptamers were selected to whole bacterial cells of non-pathogenic strain *E. coli* K12 by using a centrifugation method described by Hamula *et al*. (2008) with some modifications. Bacterial cells have different structures on their surface such as proteins and oligosaccharides. Therefore it is not clear what the aptamer target molecules are. Nine rounds of selection and counter selection after selection round 5 and 8, were performed. This was done so that only the aptamers which bind specifically to the target and not to the other bacteria can be obtained (Cao *et al*., 2009). Agarose gels after the selection round 1, 2, 3 and 4 are presented in Figure 3.5. The aptamer pools can be seen in the gel images in lanes 1 and 2 as 100 bp or slightly bigger bands. It has previously been discussed that the PCR product size may

look bigger on a gel (3.3.1). No amplification can be seen in DNA control samples where no bacterial cells were added (lanes 3), as expected. This is because the aptamers have been washed off from the samples as there are no binding sites for them in the solution. This also shows the aptamers are not binding to anything else such as the tube wall. Also, the bacterial control samples in lanes 4 are clear. This shows *E. coli* K12 has no DNA sequence for the PCR primers used in this experiment to bind and therefore cannot be amplified in PCR. The PCR control samples in lanes 0 have no 100 bp bands, only a faint band (smaller than 50 bp) that is a primer dimer, as expected.

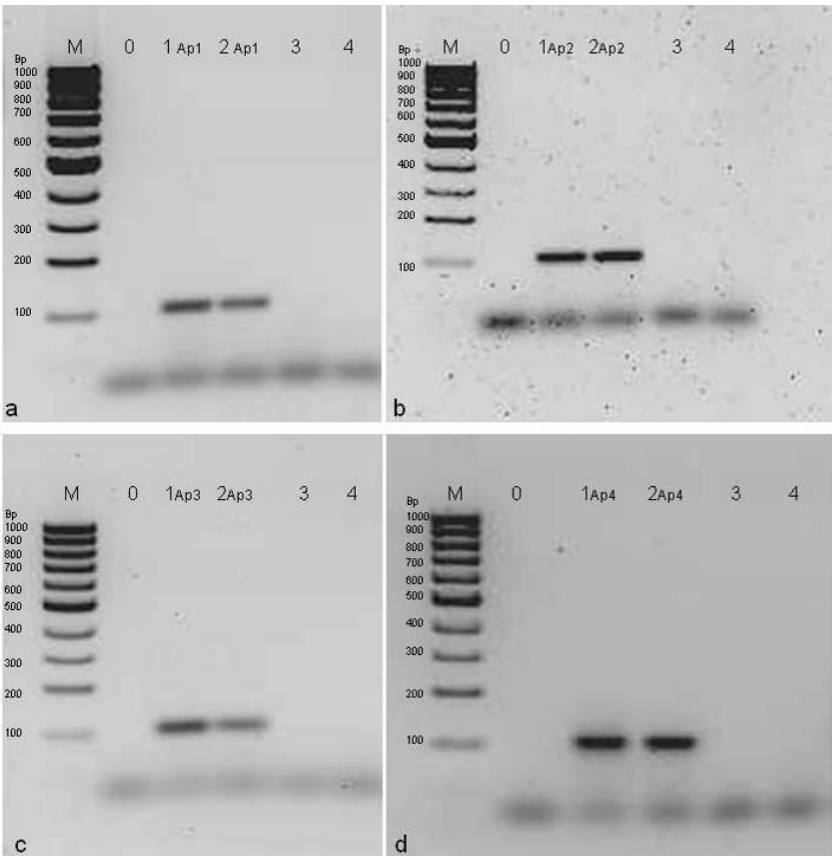


Figure 3.5 Agarose gel (2%) with aptamer pool 1 (a), 2 (b), 3 (c), and 4 (d) with two replicates. Lanes M on the gel pictures contain PCR Sizer 100bp DNA Ladder, lane 0 is the PCR control sample. The bacterial control samples are in lanes 3 and DNA controls in lanes 4.

After five rounds of selection, the counter selection was performed by using *L. bulgaricus* as a counter bacterium. The agarose gel pictures are presented in Figure 3.6. The PCR product of aptamer pool 5 is on gel 3.6a in lanes 1 and 2 and the

aptamer pool 5 after the counter selection is on gel 3.6b in lanes 1 and 2. The counter selection products are the aptamers that are binding *E. coli* K12 but not *L. bulgaricus.* A template control in lane 1 on gel 3.6b was performed to see if the addition of a template can be seen on an agarose gel. As expected, no 100 bp template band can be seen and this indicates that the bands seen on gel images are PCR amplified products. Lanes 0 on the gels are PCR control samples where the primers were added with no template DNA. All these control samples are clear as expected. The faint bands around 50 bp are the primers.

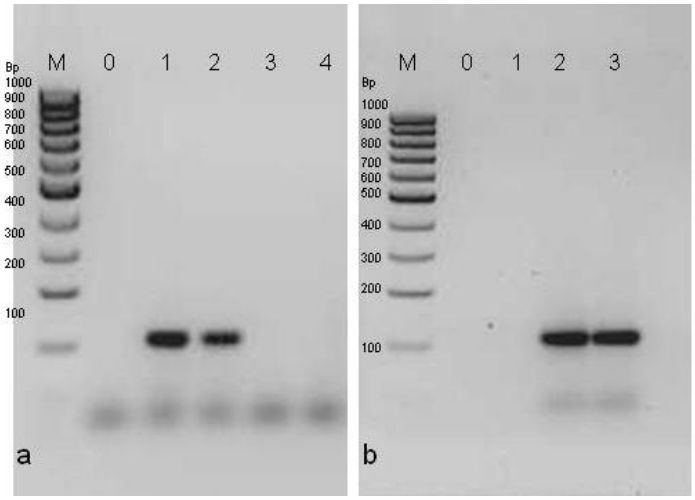


Figure 3.6 Agarose gels (2%) with aptamer pool 5 (lanes 1 and 2) before (a) and after counter selection with *L. bulgaricus* (b). Lanes M contain PCR Sizer 100bp DNA Ladder and lanes 0 are PCR control samples. On gel a the bacterial control sample is in lane 3 and DNA control in lane 4. On gel c, template control is in lane 1.

Agarose gel pictures of aptamer pools 6 and 7 are presented in Figure 3.7. The aptamer pool 6 has faint bands on the gel 3.7a. Due to the small yield of the PCR product 6, two aptamer pool 7 samples were amplified in two replicates. Samples 1.1 and 1.2, and samples 2.1 and 2.2 on gel 3.7b were mixed together after they were purified in order to achieve an aptamer pool with more aptamers. In lanes 0, where the PCR control samples are, no amplification can be seen because no template DNA was added. No amplification can be seen on bacterial control samples (lanes 3) or in DNA control samples (lanes 4), as expected. The bacterial control sample only contains bacterial cells and the DNA control samples the DNA aptamers that have been washed off because there were no binding sites for the aptamers in the mixture. The faint band, smaller than 50 bp, contains the primers.

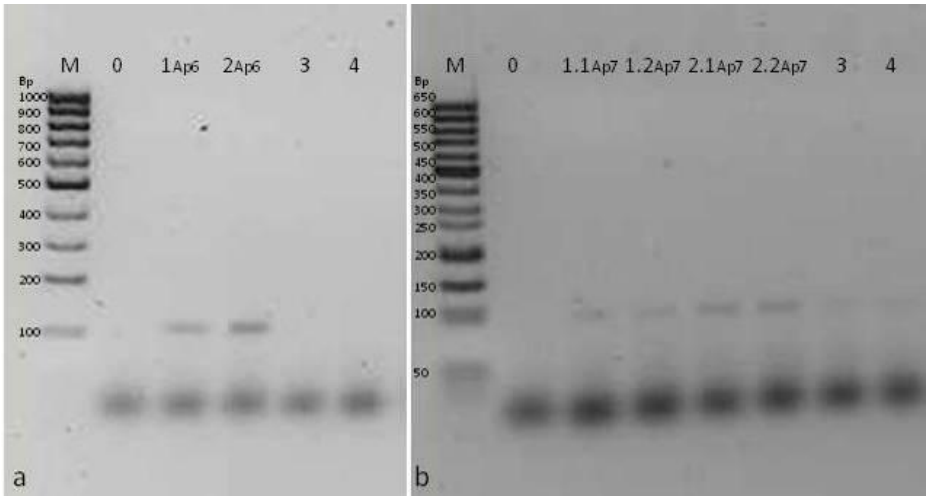


Figure 3.7 Agarose gel (2%) with aptamer pool 6 (Ap6), and 7 (Ap7). Lane M on gel a contains PCR Sizer 100bp DNA Ladder and on gel b PCR MiniSizer 50bp DNA Ladder, PCR control samples are in lanes 0. The bacterial control samples are in lanes 3 and DNA controls in lanes 4. On gel b two aptamer pools were produced in replicates (lanes 1.1Ap7, 1.2Ap7, 2.1Ap7 and 2.2Ap7).

The agarose gel pictures of the aptamer pool 8 before and after the counter selection are presented in Figure 3.8. On gel 3.8a, it can be seen that the aptamer pool samples (100 bp) are faint and therefore the samples have been amplified in two replicates (1.1, 1.2, 2.1 and 2.2). The counter selection was performed with *B. subtilis* and *S. typhimurium* and the PCR products separated on agarose gel can be seen on gel 3.8b in lanes 1 and 2. It can be seen that the intensity of the PCR products after the counter selection is much higher than before. That is because most of the aptamers in the pool are not binding to counter bacteria and therefore more template has been added to the amplification reaction. PCR control samples where only the primers have been added are in lanes 0 and no amplification products can be seen in these lanes, as expected. The faint bands smaller than 50 bp are the primers.

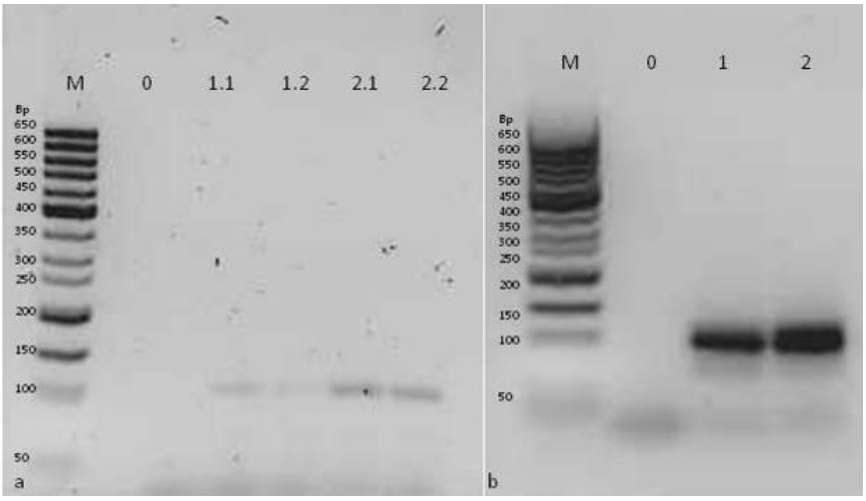


Figure 3.8 Agarose gel (2%) with aptamer pool 8 before (a) and after (b) counter selection with *B. subtilis* and *S. typhimurium*. Lanes M on the gels contain PCR MiniSizer 50bp DNA Ladder and lanes 0 are PCR control samples (no template DNA added). On gel a aptamer pool 8 is in lane 1.1, 1.2, 2.1, and 2.2. On gel b aptamer pool 8 after the counter selection is in lanes 1 and 2.

The aptamer pool 9 was selected. The number of washing steps was increased to last two selection steps in order to acquire the aptamers with high affinity (Chen *et al.*, 2007; Cao *et al.*, 2009). The PCR products were separated on polyacrylamide gel. Polyacrylamide gel was used to see if it is suitable for separating aptamer samples. The gel picture of the aptamer pool 9 and the control samples are presented in Figure 3.9. Two bands can be seen on the gel in lanes 1 and 2 around 100 bp. These samples are aptamer pool 9 in two replicates. It can be seen that the molecular weight marker (lane M) has unusual bands when comparing the bands to the other gel images above. It seems that the ladder used in this experiment is not suitable for polyacrylamide gels. The bacterial control sample in lane 3 and the DNA control sample in lane 4 are clear, as expected. The bacterial control sample only contains bacterial cells and the DNA control sample only contains the DNA aptamers that have been washed off because there were no binding sites for the aptamers in the mixture. PCR control sample, where only the primers have been added, is in lane 0. No amplification can be detected, as expected. The faint bands at the bottom of the image are the primers. The polyacrylamide gel can be used for separating the samples, but agarose gels were used in further experiments because of their overall ease of use.

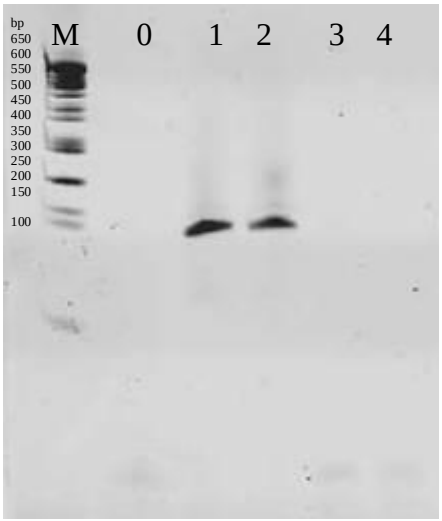


Figure 3.9 Polyacrylamide gel (10%) with aptamer pool 9. Lane M on the gel contains PCR MiniSizer 50bp DNA Ladder, lane 0 is PCR control sample. In lane 1 and 2 is aptamer pool 9. The bacterial control sample is in lane 3 and DNA controls in lane 4. The PCR was repeated 20 times.

In this study, heat denaturation was successfully used to separate the DNA stands. A problem with this technique was that when the aptamers were incubated with the target molecules, some of the ssDNA anneal back together to their complementary stands instead of forming the structure that is necessary for binding. This could have been avoided by separating the strand from each other before the incubation. It is possible to produce ssDNA molecules by using unequal length strand PCR (Cao *et al.*, 2009). When two different sized bands are produced they have different migration rates on denaturing gel electrophoresis. Also the ssDNA pool can be created by labelling one of the DNA strands with a biotin label and then separating the strands using magnetic beads (Hamula *et al.*, 2008). In the study of Hamula *et al.* (2008) the binding of the aptamers was better when the molecules were heat separated because more strands were available in the solution. Strand separation requires additional washing steps and that can cause a loss of molecules. They demonstrated the binding being better when the aptamers were heat denatured but the aptamers were more specific when they were strand separated. The heat denaturation was used in this study because of its relative simplicity and rapidity.

There are several variations of the SELEX depending on the size and the type of the target molecules (1.3.1). Aptamers have previously been selected to bind to lysozyme using a method based on filtration (Cox & Ellington, 2001). Lysozyme

was biotin-labelled and then captured to streptavidin beads. The aptamers were incubated with the beads and vacuum filtering was used to separate the non-binding molecules. The beads were recovered from the filter and the bound aptamers were partitioned from the beads by denaturing the RNA by heat. The filter technique was first used for aptamer selection in this study and was found to be overtly complicated. The elution of the bound aptamers was difficult, as boiling elution buffer with high urea concentration was needed (Vivekananda & Kiel, 2006). It was not practically possible to add boiling buffer on the filter. The other major problem occurred when the elution buffer was boiled for too long and some of the water was evaporated. This led to a precipitation of the urea on a filter making the aptamers impossible to elute. The EDTA in the elution buffer keeps the DNA more stable when stored but the DNA had to be precipitated, purified and re-dissolved in another buffer for next steps of the selection. Even though Vivekananda & Kiel, (2006) successfully selected anti-*F. tularensis* DNA aptamers by using the filtering method, in this study the elution step was found to be complicated. It can be considered that a combination of these two techniques would have resulted in an easy selection method. Once the nonbinding molecules are separated, the bacterial cells can be collected from the filter and the bound aptamers can be released from the bacteria or used directly as a template in PCR. This way the problematic elution step could have possibly been avoided. In this study the centrifugation method was found to be easy and relatively fast method to select the aptamers when the number of the samples was small.

In this study aptamers were selected to bind to whole bacterial cells and therefore it is not known what molecules the aptamers are binding. If necessary, the binding sites of the aptamers can possibly be determined by extracting the surface molecules and testing the aptamer binding against these molecules. A problem with extracting the surface molecules is that the purification might change the structure of the molecule as discussed by Hamula *et al.* (2008). Also the protein extraction is an extra step in the selection process. The purified molecules are often small and have to be bound to a solid phase support such as a gel bead in order to do the partitioning. This might change the structure or the charge of the molecules and result in aptamers that do not bind to these molecules when they are in their natural conformation on the surface of the bacterial cells. The other consideration is that some of the structures, such as ion

channels, penetrate through the whole membrane and therefore the aptamers should only be selected to bind to the side of the molecule that appears outside of the bacterial cells instead the parts that are inside the cells.

3.3.3. Biotin labelled *E. coli* K12 binding aptamers

Detection of bound aptamers can be achieved using biotin labelled aptamers (Bruno & Kiel, 1999; Vivekananda & Kiel, 2006). The biotin-labelled aptamer pool 9 was produced by PCR using biotinylated primers. The PCR products were separated on agarose gel to see the size of the products. In figure 3.10 biotin-labelled aptamer pool 9 selected to bind *E. coli* K12 is presented. Aptamer pools are the 100 bp bands in lanes 1-12. In lane 0 the PCR control sample where no template DNA was added is clear, as expected. Samples were purified with spin columns and used for the binding reaction.

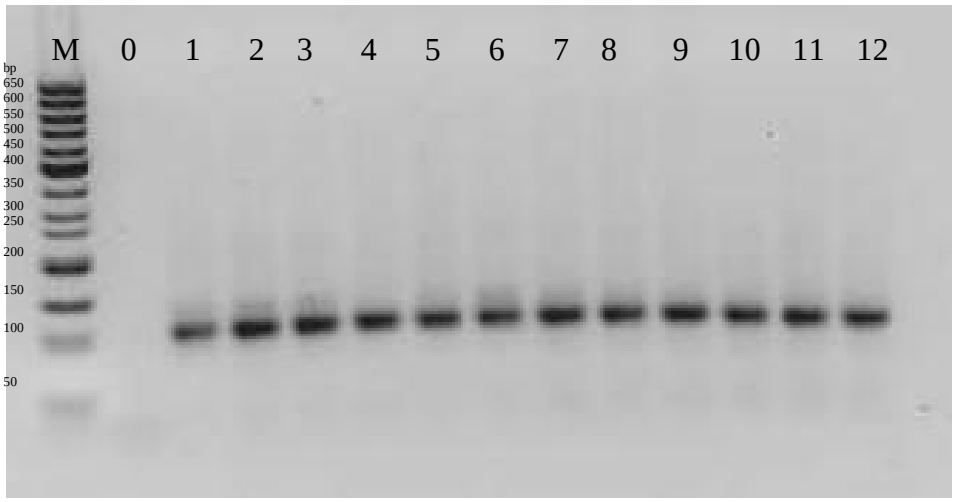


Figure 3.10 Agarose gel (2%) with biotin-labelled aptamer pool 9. Lane M is PCR MiniSizer 50 bp DNA Ladder, lane 0 is a PCR control sample and lanes 1-12 are the PCR amplified biotin-labelled aptamer pool 9.

3.3.4. Detection of *E. coli* K12 binding aptamers by enzyme linked technique

Detection of *E. coli* K12 bound aptamers was performed by using an enzyme linked technique. The ninth aptamer pool was biotin labelled. Peroxidase labelled streptavidin that binds biotin was used to detect bound aptamers. Figure 3.11 shows the absorbance of biotinylated aptamers with streptavidin peroxidase. Five different aptamer concentrations were tested in triplicate. The higher the absorbance the greater the number of aptamers have bound to the bacterial cell surface. The result

shows the aptamers have bound to the live *E. coli* K12 cells. When the aptamers have been added, there is a significant difference in the absorbance (F=73.8, P=1.4×10^{-8}). The most diluted sample, where the absorbance was significantly higher than the sample with no aptamers (0), was sample 1/40 (F=20.6, P=0.01). The method was time consuming and not very reliable due to the several washing steps that washed out too many bacterial cells from the solution. In the sandwich aptamer linked immunosorbent assay (ALISA) method developed by Vivekananda & Kiel (2006), the target molecules were captured on the microtiter plates. Therefore it can be assumed that they did not have a problem with washing off too many molecules during the washing steps.

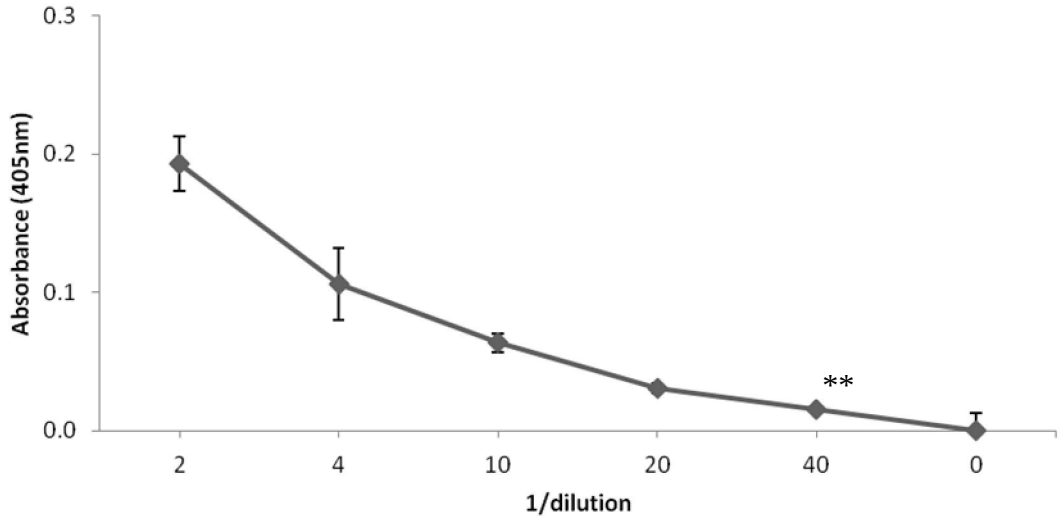


Figure 3.11 Detection of *E. coli* K12 binding biotinylated aptamers by streptavidin-peroxidase (1 µg/ml). Absorbance (405 nm) was measured after the substrate addition in triplicate (n=3). The values presented are means ± s.d. Absorbance was corrected for background (Abs 0.10). ** Highly significant (P<0,01).

3.4. CONCLUSONS

A development of aptamer selection method for whole live bacterial cells was described in this study. Aptamers were selected to bind specifically to whole live *E. coli* K12. A random 100 nucleotides long DNA library containing a 40 nt long random sequence was first produced and a technique to select the aptamers was developed. The first technique used was based on filtration (Vivekananda & Kiel, 2006) but was then replaced with an easier and faster centrifugation technique (Hamula *et al*., 2008). Counter selection was performed to exclude the aptamers that

are binding to other bacterial cells (Hamula *et al.*, 2008; Cao *et al.*, 2009). The binding of the selected aptamers was demonstrated by using an enzymatic detection.

In conclusion the detection of the bound aptamers with the enzymatic method used in this study was time consuming and the loss of the samples was great due the several washing steps this method requires. This also caused a variance in the results. In response to these observations development of an easier and more rapid method for detection of aptamer binding will be developed. The detection of the aptamers from a food matrix will be tested and finally the aptamers will be identified. The identification will be done by cloning the aptamers and sequencing the clones. The selection method developed in this study will be used to select the aptamers to bind to food poisoning bacteria.

CHAPTER 4 – Development of a fluorescence-based detection method for *Escherichia coli* K12 binding aptamers

4.1. INTRODUCTION

A method for selecting aptamers against live bacterial cells was successfully developed (3.3.2). The aptamers were selected to bind to non-pathogenic *E. coli* K12 by using a method based on centrifugation (3.3.2). The binding of these aptamers to their target was demonstrated with an enzyme-linked method (3.3.4), where the aptamers were first labelled with biotin. As the strong binding between streptavidin and biotin is well known, peroxidase-labelled streptavidin was attached to biotin. Addition of a substrate led to formation of a coloured product when reacting with the peroxidase. This colour change correlated with the amount of aptamer molecules bound to the bacterial cell surface. This method demonstrated aptamer binding to its target, but was time consuming due the number of washes. Also too many bacterial cells were washed off during the wash step leading to variable results when the method was repeated.

Aptamers can be labelled with fluorescent labels (Hoffmann *et al.*, 2007; Cao *et al.*, 2009; Joshi *et al.*, 2009). In this study, aptamers were labelled with a fluorescent 6-carboxyfluorescein (FAM) and detection methods for the binding of the FAM-labelled aptamers were developed. The binding properties of the aptamers were characterised and the specificity tested using a combination of fluorescence microscopy and fluorimetry. As *E. coli* K12 is a rather easy bacterium to work with, an alternative microscopy-based method to detect aptamer binding to smaller bacterial cells was also tested. This method was based on fluorescent-labelled streptavidin beads. These beads can bind to biotin labelled-aptamers (3.3.3) and can then potentially be used to test the aptamer binding to smaller and faster moving bacterial cells such as *Salmonella*. This study demonstrates that the *E. coli* K12 aptamer pool bound specifically to its target.

4.2. METHODS

4.2.1. Development of a fluorescence detection method for *E. coli* K12 binding aptamers

4.2.1.1. FAM-labelled aptamers

Aptamers were selected to bind to *E. coli* K12 (3.2.2). The aptamer pools used in the experiments were labelled with 5' fluorescence (FAM) label (2.3.7) and by monitoring the label the aptamers can be detected. For all the experiments, aptamer pools were produced by PCR using the aptamer pool 9 as a template. More template was produced by amplifying the aptamer pool 9 PCR product in dilution 1:40 (Nested template) by PCR. The PCR amplified aptamer pools were separated on agarose gels and the images were taken followed by a purification of the product by spin columns. The PCR product concentration was measured using a spectrophotometer to measure the absorbance of the samples at a wavelength 260 nm.

4.2.1.2. Fluorimetry

Fluorescence values of the *E. coli* K12 bacterial cells when FAM-labelled aptamers were bound to them were measured to detect the aptamer binding. *E. coli* K12 binding aptamers with FAM-labels were incubated with bacterial cells (2.3.9.1) at three different concentrations (10 pmol, 20 pmol and 30 pmol). Samples were incubated at room temperature for one hour and the samples were washed with binding buffer (BB). The fluorescence of the samples was measured with a fluorescence plate reader (2.3.9.3) and the fluorescence values were compared by one way analysis of variance (ANOVA).

4.2.1.3. Fluorescence microscope

The fluorescence microscope was used to visualise the FAM-labelled aptamers bound to bacterial cell surface. *E. coli* K12 binding aptamers with FAM-labels were incubated with overnight grown *E. coli* K12 culture (2.3.9.1) and the samples were visualised under a microscope (2.3.9.2). Two different aptamer concentrations (10 pmol and 50 pmol) were used and a control with no aptamers. The images were taken from six random fields with green (495 nm) and visible light and the fluorescent labelled bacterial cells were counted from the images. Results were compared by one way ANOVA.

4.2.2. Optimal binding time of the aptamers

FAM-labelled aptamer pool 9 specific for *E. coli* K12 was used to measure the optimal binding time of the aptamers. The binding reaction (2.3.9.1) was performed with aptamer pool 9 (10 µl, approximately 6 pmol) and 90 µl of an overnight *E. coli* K12 culture. The samples were incubated at room temperature in triplicate for 0, 15, 30, 45, 60, and 75 minutes and the fluorescence values were measured with a fluorescence plate reader (2.3.9.3). The samples were washed three times with 200 µl of BB and the fluorescence was measured from the washes in order to find out the number of washes needed to wash off the non-specifically binding aptamers. The fluorescence values were compared by one way ANOVA.

4.2.3. Binding of the aptamer pool 3, 5, 7 and 9

Binding of the aptamer pools was tested in order to see their binding capacity. Aptamer pools were collected after each round of aptamer selection (3.2.2) and the FAM-labelled aptamer pools were produced by PCR. Binding properties of the aptamer pools 3, 5, 7 and 9 were tested by incubating the FAM-labelled aptamers with bacterial cells (2.3.9.1) and measuring the fluorescence of the samples. Aptamers were incubated in 50 µl of *E. coli* K12 suspension with three aptamer concentration (10 pmol, 15 pmol and 25 pmol) for aptamer pool 5 and 9. Due the small number of aptamers (PCR product), only two different aptamer concentrations (10 pmol and 15 pmol) were used for the analysis of aptamer pool 3 and 7 with no replicates. After 45 minutes incubation at room temperature the bacterial cells were washed and the fluorescence of the samples was measured with a fluorescence plate reader (2.3.9.3).

4.2.4. Specificity of the *E. coli* K12 binding aptamers

The specificity of the aptamers was tested in order to see if the *E. coli* K12 binding aptamers are specific to *E. coli* K12 or if they bind to other bacteria too. FAM-labelled aptamer pool was incubated with different bacterial cells. Two different types of experiments were performed. The samples were visualised under a fluorescence microscope and the fluorimetry experiments were performed. The aptamers were also tested to see if *E. coli* K12 could be detected from a mixture of bacterial cells. No replicates were performed for the fluorescence experiments because only a small number of aptamers was available.

4.2.4.1. Binding of the aptamers to *E. coli* B, *B. subtilis* and *S. aureus*

The *E. coli* K12 specific aptamer pool 9 was tested with *E. coli* B, *B. subtilis* and *S. aureus*. *E. coli* K12 was used as a positive control. Bacterial suspensions were prepared (2.3.9.1) and 50 µl of this suspension was incubated with three different amounts of aptamers. *E. coli* K12 was incubated with 10 pmol, 20 pmol and 30 pmol of aptamers and the other strains (*E. coli* B, *B. subtilis* and *S. aureus*) with 5 pmol, 20 pmol and 30 pmol of aptamers. 5 pmol of aptamers were used instead of 10 pmol, because not enough aptamers were produced (small PCR yield). A negative control sample, where no aptamers were added, was made for all different bacteria samples. The fluorescence was measured from all of the samples by a plate reader (2.3.9.3). The microscope images were taken (2.3.9.2) from the 20 pmol samples (*E. coli* K12, *E. coli* B and *S. aureus*) with a green fluorescence light and visible light from five random fields and the fluorescent labelled bacterial cells were counted. The fluorescence values were compared by one way ANOVA

4.2.4.2. Detection of *E. coli* K12 from a bacterial mixture with FAM-labelled aptamers

The aptamer pool nine was tested to see if the aptamers are able to detect the *E. coli* K12 bacterial cells from a mixture of different bacterial cells. The bacterial mixture containing equal amounts of *E. coli* K12, *E. coli* B and *S. aureus* was prepared. Each bacterial suspension was prepared as described (2.3.9.1) and each suspension was mixed together to a total sample volume of 100 µl. The control samples were prepared by adding a third of bacterial suspension and topped up to 100 µl with BB. The aptamers (20 pmol) were incubated with the bacterial cell suspension followed by the washes. No replicate samples were done. The fluorescence was measured by a plate reader (2.3.9.3).

4.2.4.3. Binding of the aptamers to *L. acidophilus*

L. acidophilus is a common bacterium found in dairy products such as yogurt and was therefore chosen to be one of the strains to be tested. The specificity experiment (4.2.4.1) was performed by incubating 20 pmol of FAM-labelled aptamers with a *L. acidophilus* strain. *E. coli* K12 was used as a positive control. The negative control samples, with no added aptamers, were made for both strains. The fluorescence values were measured by a fluorescence plate reader (2.3.9.3).

4.2.5. Fluorescent microspheres

A method to visualise the biotin labelled aptamers (2.3.7) binding with fluorescent labelled NeutrAvidin microspheres (FluoSpheres) was developed (2.3.10). The method was developed in order to detect aptamers binding to some other bacterial cells that are smaller than *E. coli* K12 and therefore difficult to see under the fluorescence microscope using FAM-labelled aptamers. The FluoSpheres are small fluorescent NeutrAvidin spheres, and therefore have a high affinity to bind to biotin, as stated before for streptavidin (2.3.8). Biotin-labelled aptamers (50 µl) (eight PCR products in 100 µl spin column elution buffer) were incubated with *E. coli* K12 bacterial cells. The samples were washed and the FluoSpheres were added and incubated (2.3.10). Non-binding FluoSpheres were washed off and the samples were visualised under a fluorescence microscope with a green fluorescence and visible light.

4.2.6. Aptamer detection of live and dead *E. coli* K12 bacteria cells

The Live/Dead *Bac*Light staining was introduced to see if the *E. coli* K12 specific aptamers are binding to live or dead bacterial cells (2.3.11). The FAM-labels of the aptamers were difficult to see on the microscope images because of the brighter fluorescence of the Live/Dead *Bac*Light staining. Instead of using the FAM-labelled aptamers the FluoSpheres were used to detect the bound aptamers. The biotin labelled aptamer pool was first incubated with *E. coli* K12 as previously described (4.2.5). Once the aptamers and the fluorescence microspheres were bound to the bacterial cell surface the Live/Dead staining was performed (2.3.11). The samples were visualised by a fluorescence microscope and the images were taken.

4.3. RESULTS AND DISCUSSION

4.3.1. Development of fluorescence detection method for *E. coli* K12 binding aptamers

4.3.1.1. FAM-labelled aptamers

Aptamers can be labelled with different fluorescent dyes. Green dyes such as 6-carboxyfluorecein (FAM) (Joshi *et al.,* 2009) and fluorescein (FITC) (Wang *et al.,* 2003; Hoffmann *et al.* 2007; Cao *et al.*, 2009) are commonly used. The binding of these aptamers can be detected with fluorescence-based methods such as

fluorescence microscopy (Ohk, *et al.*, 2010) and flow cytometry (Wang *et al.*, 2003; Hoffmann *et al.*, 2007; Cao *et al.*, 2009; Terazono *et al.*, 2010). In this study, aptamers were produced for all the different experiments with FAM-labelled primers by PCR and the samples were separated on an agarose gel followed by purification of the samples. Figure 4.1 shows an agarose gel with the PCR products of the fluorescent labelled aptamers in lanes 1-7. By comparison with a molecular weight marker in lane M, it can be seen the sizes of the aptamers are around 100 bases, as expected. The PCR control (no template DNA) sample in lane 0 is clear as expected. The PCR products were purified by using spin columns.

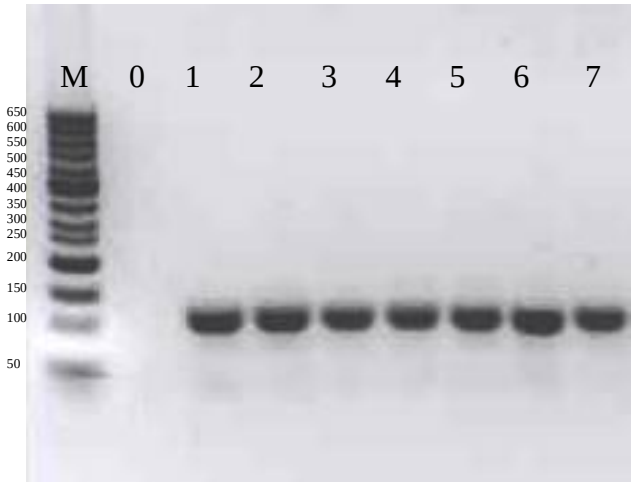


Figure 4.1 Agarose gel (2%) with FAM-labelled *E. coli* K12 binding aptamer pool 9. Lane M on the gel contains PCR MiniSizer 50 bp DNA Ladder, lane 0 is a PCR control sample (no template DNA added) and FAM-labelled aptamers with an aptamer pool 9 are in lanes 1-7.

4.3.1.2. Fluorimetry

The detection of *E. coli* K12 binding FAM-labelled aptamer pool 9 was performed by fluorimetry using a fluorescence plate reader. The fluorescence of the *E. coli* K12 samples incubated with different aptamer concentrations (10 pmol, 20 pmol and 30 pmol) are shown in Figure 4.2. The greater the number of bound aptamers the higher the fluorescence can be seen in the figure. It can be seen that when the aptamers have been added the fluorescence values are significantly higher (F=71.85, p=3.98×10^{-6}). The results show that 10 pmol addition of the aptamers is enough to detect the *E. coli* K12 bacterial cells (F=81.8, p=8.2×10^{-4}). These results show that the aptamers have bound to live *E. coli* K12 cells. This method developed for aptamer detection is easy

to perform and repeatable. The technique will be used in further aptamer characterisation experiments.

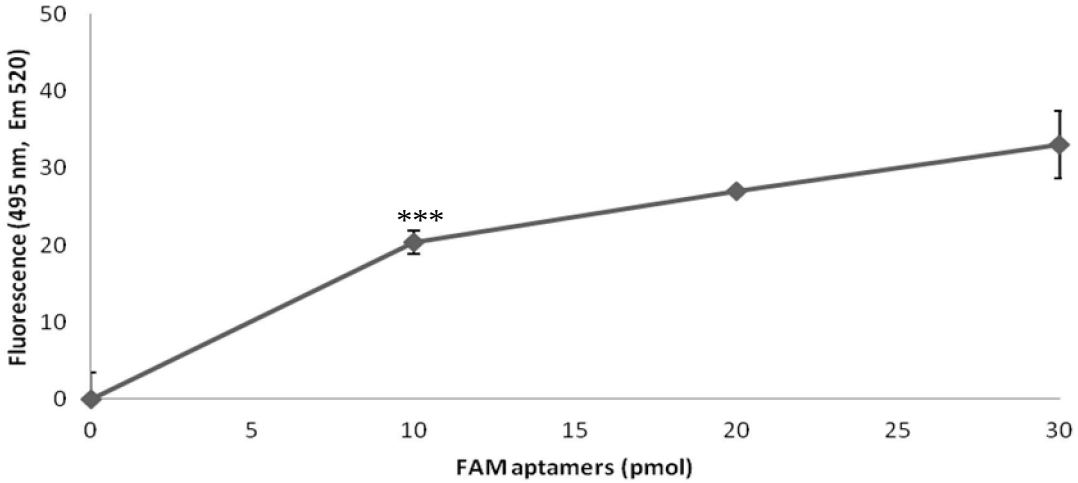


Figure 4.2 FAM-labelled aptamer pool 9 binding to *E. coli* K12. The fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (10.0). *** Very highly significant (P<0.001).

4.3.1.3. Fluorescence microscope

Fluorescence microscopy has previously been used to visualise the aptamer binding (Ohk *et al.*, 2010). In this study, the aptamers were incubated with the *E. coli* K12 bacterial cells and a microscope was used to visualise the bacterial cells with aptamers bound to them. The images were taken from each field with fluorescence and visible light. Images of negative control samples with no added aptamers (0 pmol) and samples with 10 pmol and 50 pmol aptamers are shown in Figure 4.3. The results show that when FAM-labelled aptamers are not added (0 pmol) (left hand side), no fluorescent dots can be seen but the dots can be seen when aptamers are added (10 pmol and 50 pmol), as expected. This indicates that the aptamers have bound to the live *E. coli* K12 cells. By comparing the fluorescence images (left hand side) to the images taken with a visible light (right hand side), one can see that there are more dots on the light microscope images that on the fluorescence images that have been taken from the same field. It could be possible that the aptamers used bind to cells which may be in a specific stage of the cell cycle.

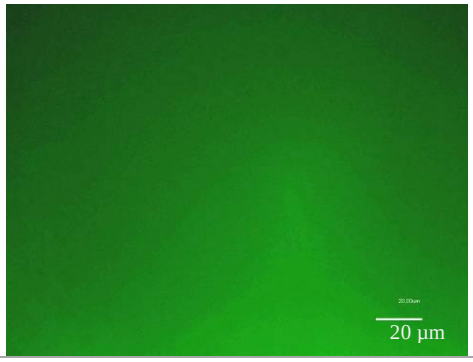
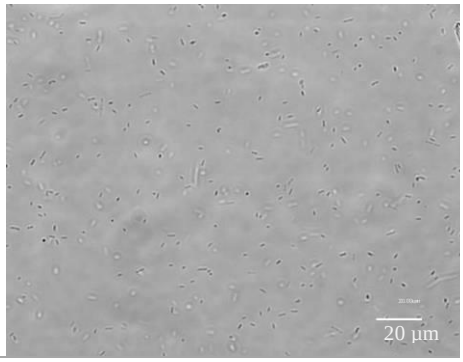
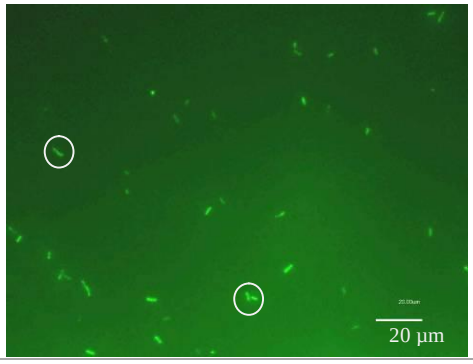
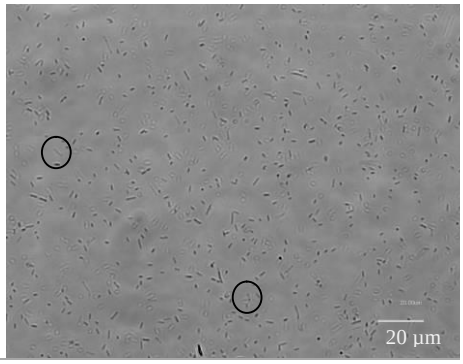
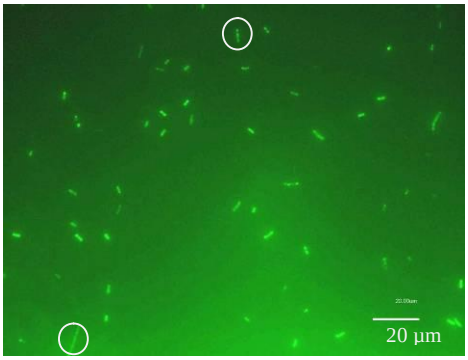
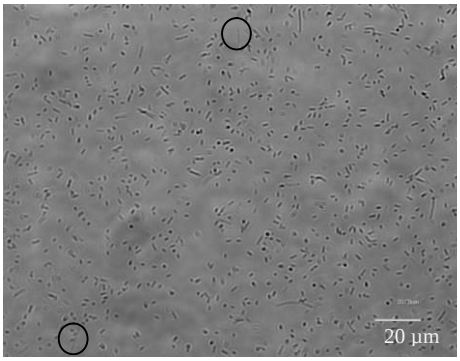
Aptamer pool 9 (**pmol**)	Fluorescence, green light (495 nm)	Visible light
0		
10		
50		

Figure 4.3 FAM-labelled aptamer pool 9 binding to the surface of *E. coli* K12. Images were taken with a fluorescence microscope with 60× magnification with a green light (495 nm) and visible light from the same field. No aptamers were added to the control sample (0 pmol) while 10 pmol and 50 pmol aptamers were added to the samples. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

An enlarged fluorescence microscopy image of the bound aptamers is presented in Figure 4.4. The long structures can often be seen in the fluorescence images taken in this study. This structure may indicate that the cells, which are detected with the aptamers, are in a dividing stage.

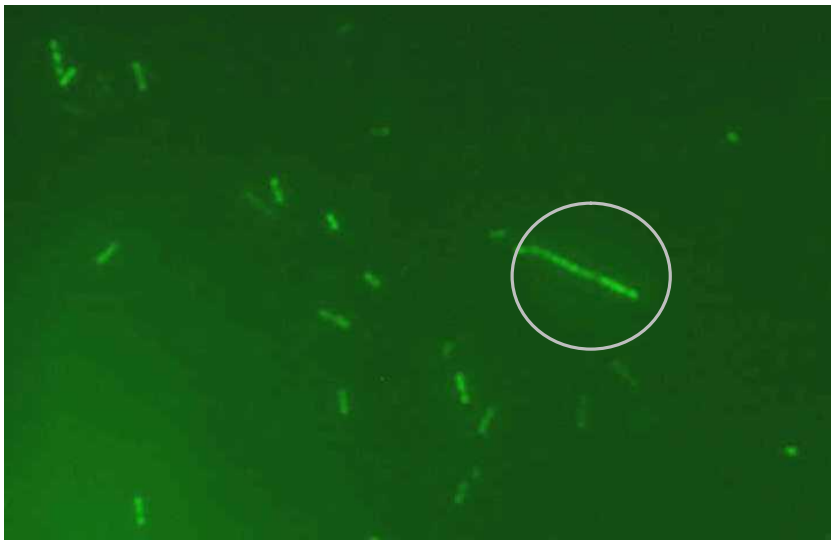


Figure 4.4 FAM-labelled aptamer pool binding to the surface of *E. coli* K12. Image was taken with a fluorescence microscope with 60× magnification with a green light (495 nm). The long structure circled was a typical finding in fluorescence images.

4.3.2. Optimal binding time of the aptamers

Optimal binding time of the aptamers was measured by incubating the aptamer pool 9 with *E. coli* K12 and measuring the fluorescence after different time points. The first sample collected was after 0 minutes of incubation and then samples were collected every 15 minutes until 75 minutes. The samples were washed three times before the fluorescence was measured. The fluorescence values are presented in Figure 4.6. It can be seen that the highest fluorescence values is achieved after 45 minutes incubation. Next sample collected (60 min) shows a lower fluorescence. For the 75 min sample the fluorescence increased again back to the same level with the 45 min sample. The lower fluorescence in 60 min sample may be due to a loss of bacterial cells in one of the replicates during the washes and therefore the measured fluorescence is slightly lower. Also the aptamer concentration may have been lower in this sample than in the other samples. However, there is no significant difference between the samples 45 min, 60 min and 75 min (F=1, p=0.4). It can also be seen

that no significantly higher binding was detected when the samples were incubated for 15 minutes (F=2, p=0.2) but 30 min incubation was enough to see significant increase in the binding (F=12.5, p=0.02). Incubation time 45 minutes was routinely used in following experiments.

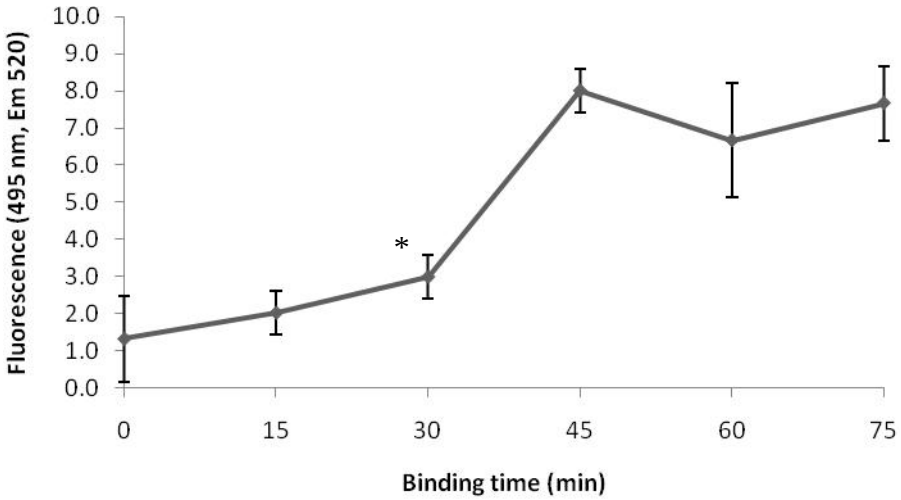


Figure 4.5 Optimal binding time of the aptamers. FAM-labelled aptamer pool 9 (~6 pmol) was incubated with bacterial cells and the fluorescence (495 nm, Em 520) was measured by a plate reader after 0, 15, 30, 45, 60 and 75 min in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (9.33). * Significant (P≤0.05).

The washes of the optimal binding time samples (0, 15, 30, 45, 60 and 75 min) were collected and the fluorescence was measured to determine the number of washes needed to wash off non-binding or aptamers with less affinity. The fluorescence values for non-binding aptamers after the first wash are presented in Figure 4.7, and after the second and third wash in Figure 4.8. It is noticeable that the fluorescence values are very high (fluorescence 290-330) after the first wash (Figure 4.7) comparing to the second or third washes (fluorescence less than 4) (Figure 4.8). This indicates most of the non-binding aptamers are washed off after the first wash and three washes is enough to wash off the non-binding aptamers. In Figure 4.8, it can be seen that the 60 min sample has a lower fluorescence than the 45 min or 75 min sample. As stated above, it is possible the actual aptamer concentration added to the sample has been lower. It can also be seen that the fluorescence of the first wash (Figure 4.7) is much higher than the fluorescence of the actual samples where the aptamers have bound to the *E. coli* K12 (Figure 4.6). There can be several reasons

for such a high number of non-binding aptamers. It is possible that the most of the denatured DNA aptamers renatured back to their double stranded form with their complementary strands. This would be instead of forming the structure that allows aptamers to bind to their target when mixed with the bacterial cells. Another reason for the low binding of the aptamers could be that there were not enough bacterial cells serving the binding sites for the aptamers. It has previously been demonstrated that these *E. coli* K12 binding aptamers do not detect all of the *E. coli* K12 cells in the solution (Figure 4.3). In Figure 4.7 it can also be seen that less fluorescence was detected in the 45 min sample than in the other samples. This may indicate that more aptamers were bound to the bacterial cell surface and had not been washed off. In conclusion, 45 minutes was demonstrated to be an optimal binding time for the aptamers and three washes can be used to remove non-binding aptamers from the solution. The 45 min incubation time at room temperature (Hamula *et al.,* 2008) and at 37^{o}C (Cao *et al.,* 2009) has previously been used but it has not been reported to be the optimum binding time for the aptamers against whole bacterial cells. In some protocols aptamers were only interacting with their target on a lateral flow on the chromatography device when the selection was done (Joshi *et al.,* 2009) and for 30 minutes when detecting the whole bacterial cells. A low incubation temperature 4^{o}C has also been reported for the aptamers that are targeted to intact whole cells (Terazono *et al.,* 2010). These findings indicate the aptamers can potentially be working in a wide temperature range.

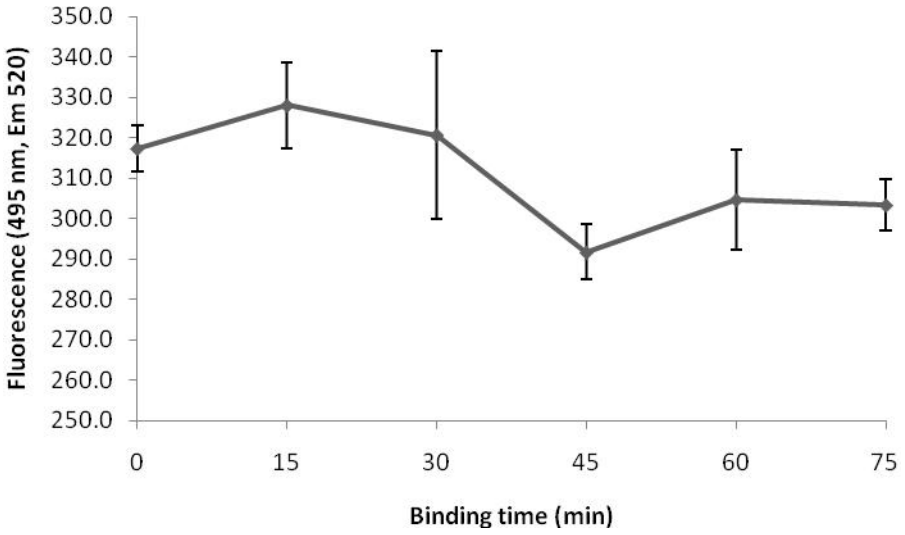


Figure 4.6 Fluorescence of non-binding aptamers after the first wash. FAM-labelled aptamer pool 9 (~6 pmol) was incubated with *E. coli* K12. Fluorescence (495 nm, Em 520) was measured after 0, 15, 30, 45, 60 and 75 min in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (0.67).

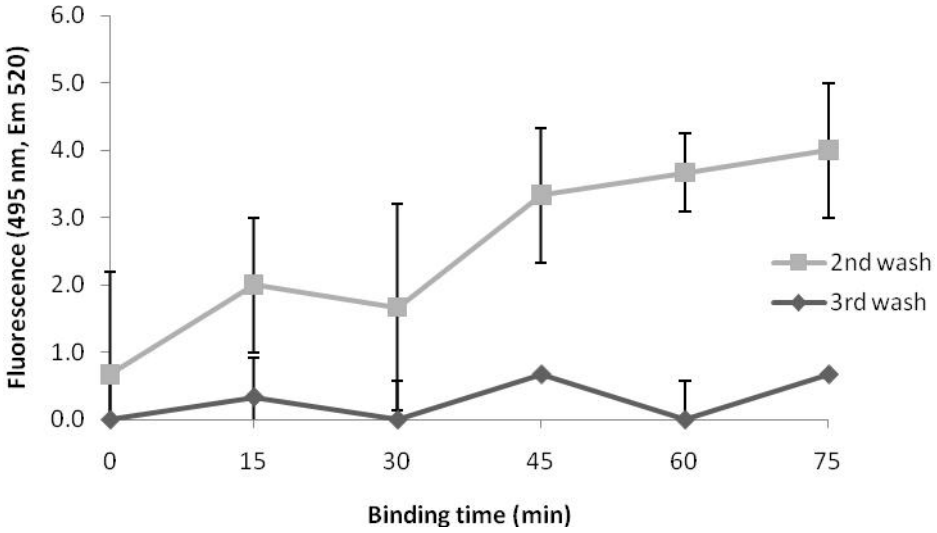


Figure 4.7 Fluorescence of non-binding aptamers after the 2nd and 3rd wash. FAM-labelled aptamer pool 9 (~6 pmol) was incubated with *E. coli* K12. Fluorescence (495 nm, Em 520) was measured after 0, 15, 30, 45, 60 and 75 min in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (1.33).

4.3.3. Binding of the aptamer pool 3, 5, 7 and 9

Aptamers were selected to bind to live *E. coli* K12 bacterial cells by repeating the selection process nine times. The binding of the selected aptamer pool 9 was previously demonstrated. The affinity of the aptamer pool to its target increases after each round of selection (Wang *et al*., 2003) but it is possible that the aptamer affinity decreases if too many rounds of selection are performed (Hamula *et al.*, 2008). In this study the binding of some of the aptamer pools was tested in order to see if the binding increased during the selection process. FAM-labelled aptamer pools 3, 5, 7 and 9 were incubated with *E. coli* K12 bacterial cells and the fluorescence of the samples was measured. The fluorescence values of each pool are presented in Figure 4.9. Aptamer pool 5 and 9 were tested with three concentrations (10 pmol, 15 pmol and 25 pmol) while aptamer pools 3 and 7 were tested with two different concentrations (10 pmol and 15 pmol). That is because the PCR yield (aptamers) was smaller for the aptamer pool 3 and 7. The PCR yield of different aptamer pools might vary depending on the quality of the template or the amount of the aptamer molecules in each sample. The results show that the highest values were obtained for pools 7 and 9. This indicates that at least seven or nine rounds of selection have to be performed in order to achieve a specific pool of aptamers. The screening should be done after each round of selection in order to find the best binding pool of aptamers. If aptamers were further selected, for example aptamer pool 10, it could be possible to obtain an even more specific pool. In this study, pool 9 was selected to be enough specific to bind to *E. coli* K12 bacterial cells. The screening was not done during the selection process because the detection method was developed after the aptamers were selected.

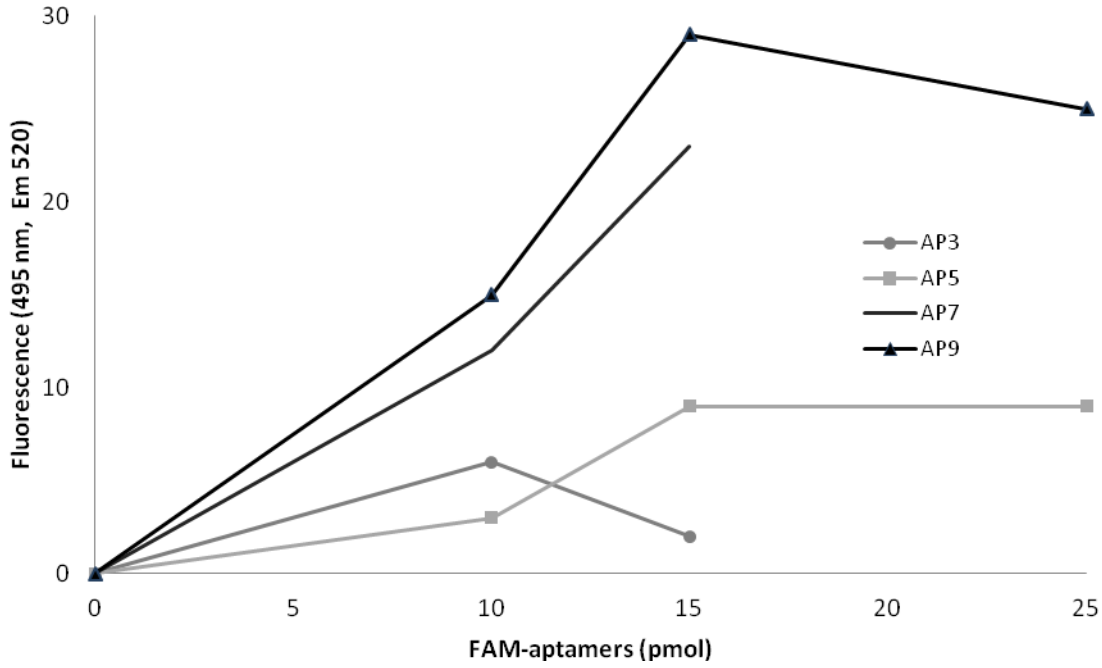


Figure 4.8 FAM-labelled aptamer pools 3, 5, 7 and 9 binding to *E. coli* K12 bacterial cells. The fluorescence (495nm, Em 520) was measured by the plate reader (n=1). Fluorescence values were corrected for background (AP3=5; AP5=3; AP7=1; AP9=1).

4.3.4. Specificity of the *E. coli* K12 binding aptamers

4.3.4.1. Binding of the aptamers to *E. coli* B, *B. subtilis* and *S. aureus*

The aptamers were specifically selected to bind to *E. coli* K12 (3.3.2) and the counter selection was done to exclude the molecules that were specific to other micro-organisms. To test the specificity of these aptamers, the binding was tested against some other bacterial cells. The fluorescent labelled aptamer pool 9 was incubated with *E. coli* K12, *E. coli* B, *B. subtilis* and *S. aureus* cultures with three different concentrations. For *E. coli* K12 10 pmol, 20 pmol and 30 pmol aptamers were used and for *E. coli* B, *B. subtilis* and *S. aureus*) 5 pmol, 20 pmol and 30 pmol aptamers were used. The fluorimetry test was performed for all samples. The fluorescence values are presented in Figure 4.10 and it can be seen that the more aptamers there are in the sample, the higher the fluorescence. The highest fluorescence values can be seen in *E. coli* K12 sample, even when small amounts (5-10 pmol) of aptamer are added. The fluorescence values for the other samples (*E. coli* B, *S. aureus* and *B. subtilis*) are very low. *B. subtilis* has given a higher fluorescence value when 30 pmol aptamers have been added. The higher fluorescence might not be actual binding to bacterial cells and could possibly have been caused by the clusters the bacteria form

while growing in broth. The aptamer concentration 20 pmol was selected to use in the following specificity experiments because it showed high fluorescence for *E. coli* K12 but not much fluorescence for *E. coli* B, *S. aureus* or *B. subtilis*.

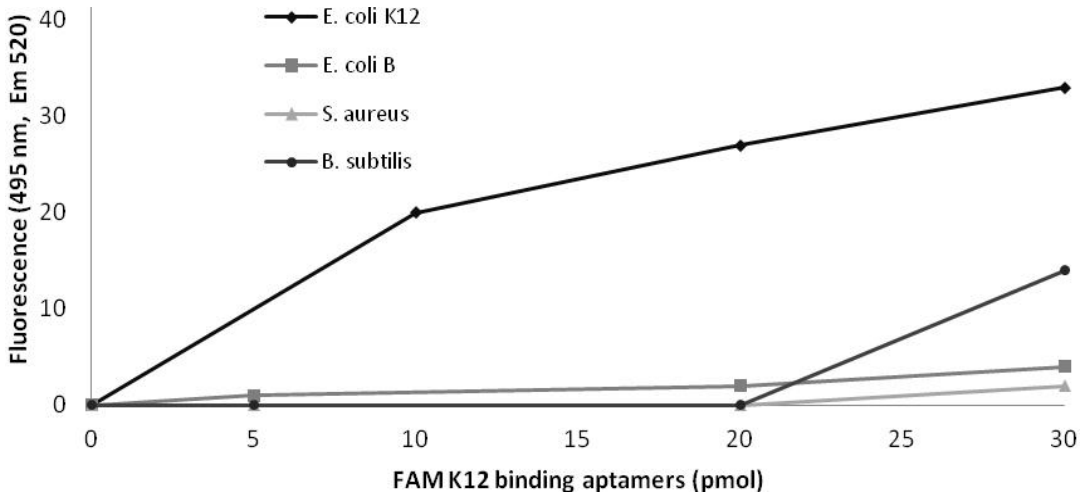


Figure 4.9 Specificity of *E. coli* K12 specific aptamer pool 9. FAM-labelled aptamers were incubated with *E. coli* K12 (positive control), *E. coli* B, *B. subtilis* and *S. aureus*. The fluorescence (495 nm, Em 520) was measured by a plate reader (n=1). Fluorescence has been corrected for background (*E. coli* K12=10; *E. coli* B=1; *S. aureus*=6; *B. subtilis*=4).

The fluorescence microscope images were taken of each sample (*E. coli* K12, *E. coli* B, *S. aureus* and *B. subtilis*) from five random fields and the fluorescent dots were counted. The images were taken with a green fluorescence light and with a visible light. The microscopy images of 20 pmol samples of *E. coli* K12 (positive control) and *E. coli* B are presented in Figure 4.11 and of *B. subtilis* and *S. aureus* are presented in Figure 4.12. It can be seen in the microscope images, when the FAM-labelled aptamers are added, that bacterial cells with fluorescent labels are visualised as green dots. When the FAM aptamers were incubated with *E. coli* B, four fluorescent dots can be detected in the image. This indicates that the aptamers might also bind to *E. coli* B, but not as strongly as to *E. coli* K12. It may be possible that the dots are not actually aptamer binding to the bacterial cell surface but are the remaining fluorescence aptamers in the solution instead. In Figure 4.12 no separate bacterial cells can be seen on *B. subtilis* samples as the bacterial cells were growing in clusters in the broth. The clusters did not break down to separate bacterial cells

when the samples were mixed and that can also be seen on the microscope images. The negative control sample (0 pmol) for *B. subtilis* looks the same as the sample where the aptamers have been added (20 pmol). Due to the difficulties of handling *B. subtilis*, it was not used in following experiments. In Figure 4.12 *S. aureus* samples are shown. It can be seen in the samples, where FAM-labelled aptamers were added (20 pmol), that some of the bacterial cells are detected with the aptamers but it can also be seen that the number of detected bacteria is much smaller compared to when the aptamers were added to the positive control samples (*E. coli* K12, Figure 4.11). This result indicates that some of the aptamers might bind to *S. aureus*, but the binding is not as strong as to *E. coli* K12. When comparing the *S. aureus* (Figure 4.12) images taken with a visible light (right hand side) to the image taken with fluorescence light (left hand side), it can be seen that there is an area where more bacterial cells are in the same place. This area can also be seen brighter green in the fluorescence image.

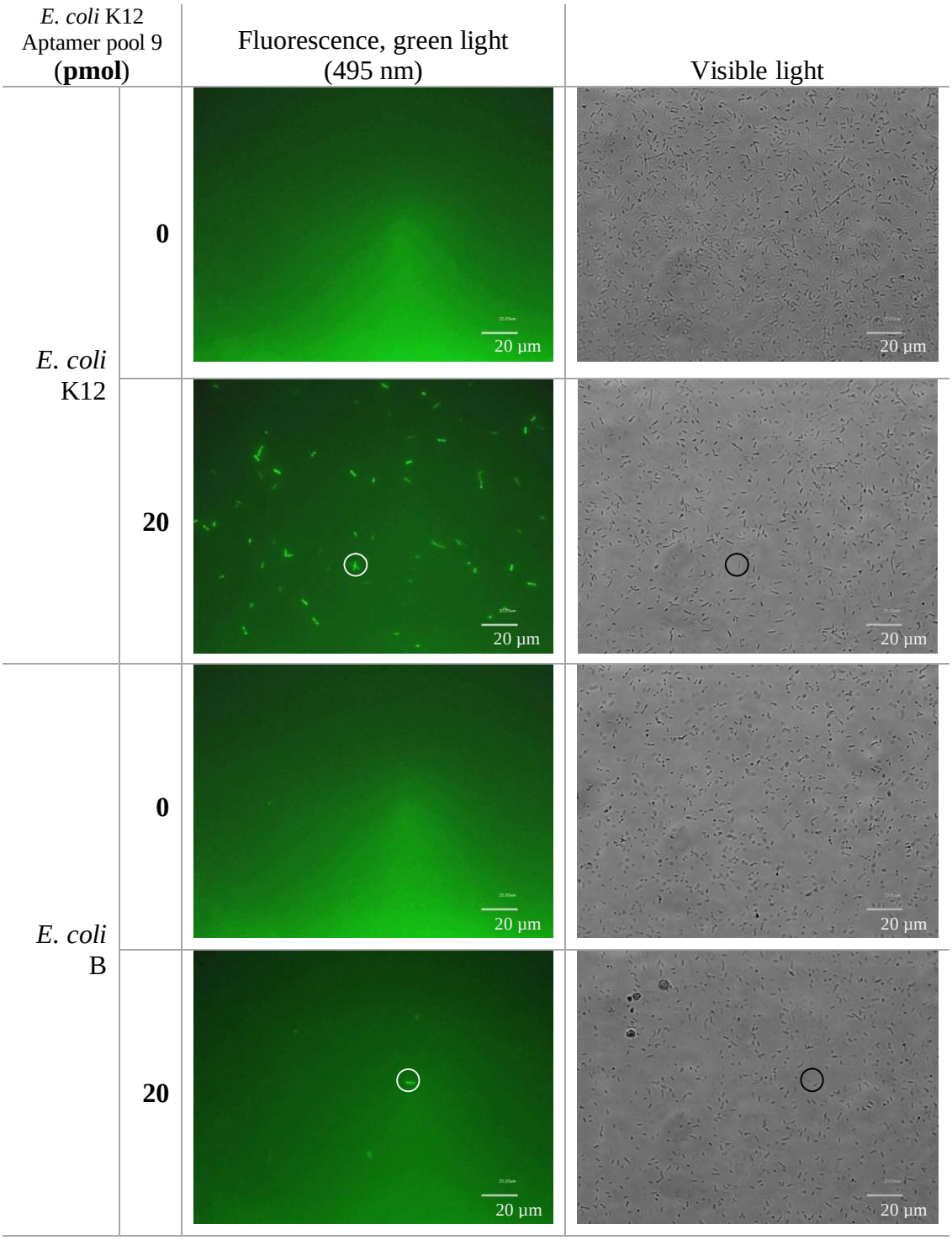


Figure 4.10 Specificity of the *E. coli* K12 binding aptamers. Aptamers were incubated with *E. coli* K12 (positive control), *E. coli* B and the images were taken with a fluorescence microscope with 60× magnification with a green light (495 nm) and visible light. No aptamers were added to the control sample (0 pmol) while 20 pmol aptamers were added to the samples. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

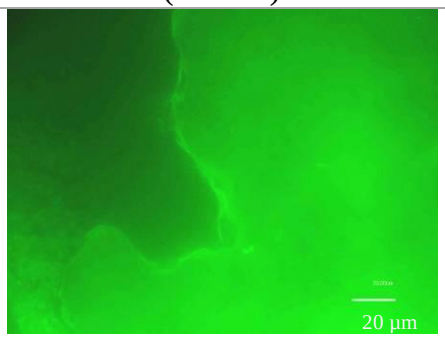
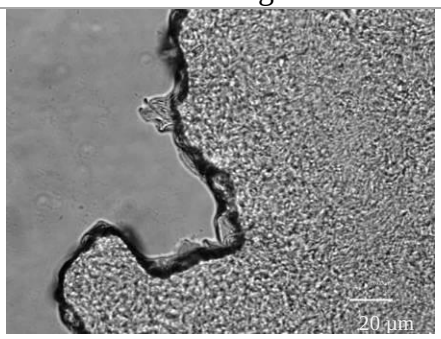
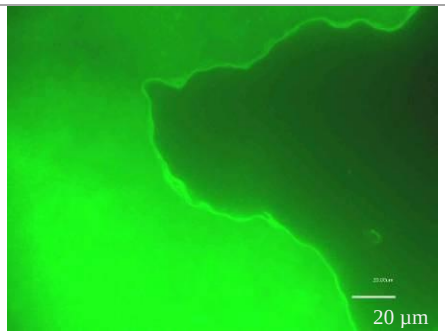
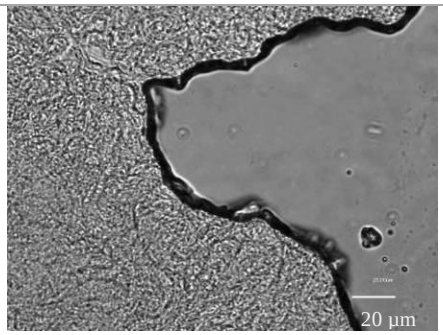
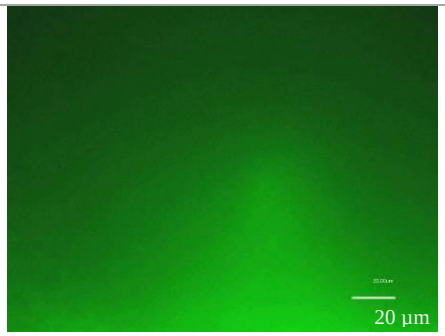
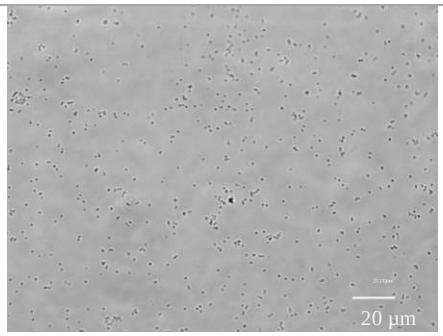
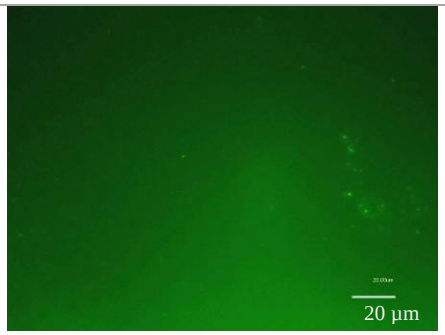
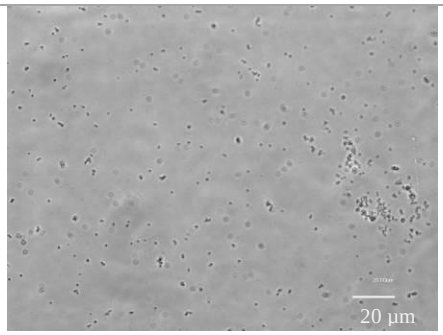
E. coli K12 Aptamer pool 9 (**pmol**)		Fluorescence, green light (495 nm)	Visible light
B. subtilis	**0**		
	20		
S. aureus	**0**		
	20		

Figure 4.11 Specificity of the *E. coli* K12 binding aptamers. Aptamers were incubated with *B. subtilis* and *S. aureus* and the images were taken with a fluorescence microscope with 60× magnification with a green light (495 nm) and visible light. No aptamers were added to the control sample (0 pmol) while 20 pmol aptamers were added to the samples.

Fluorescent labelled bacterial cells were counted from five images (n=5) of *E. coli* K12, *E. coli* B and *S. aureus*. The results are presented in Figure 4.13. It can be seen that the *E. coli* K12 samples had significantly more bacterial cells with aptamers bound to them (green dots) than the *E. coli* B and *S. aureus* samples (F=75.8, p=1.55×10^{-7}). The aptamers were specifically binding to *E. coli* K12 but very low detection levels can be seen with *E. coli* B and *S. aureus.*

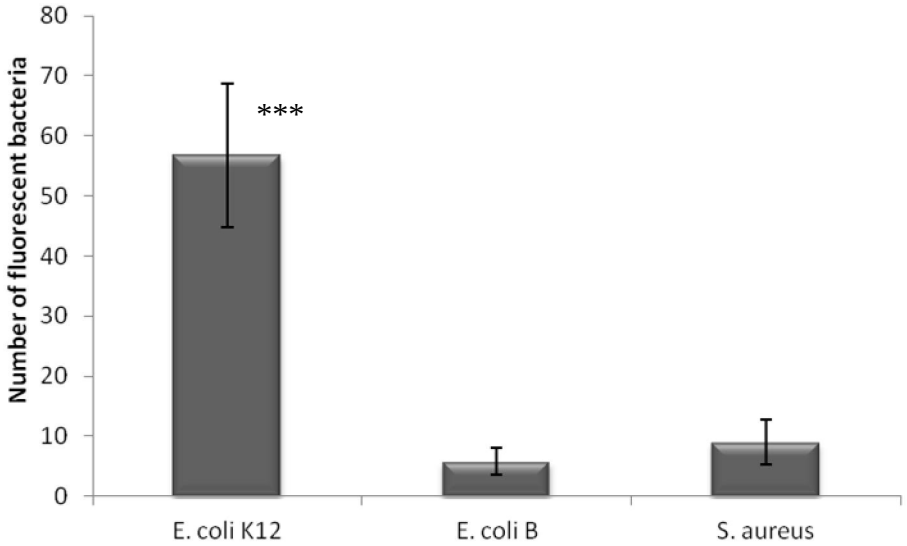


Figure 4.12 Number of fluorescent labelled bacteria. Aptamers (20 pmol) were incubated with *E. coli* K12, *E. coli* B and *S. aureus* and the fluorescence images were taken from five random fields (n=5). The green bacterial cells were counted from the images. The values presented are means ± s.d. *** very highly significant (P<0.001).

4.3.4.2. Detection of *E. coli* K12 from a bacterial mixture with FAM-labelled aptamers

Many food products, such as cheese and yogurt, naturally contain non-pathogenic micro-organism. Therefore it is very important that the aptamers can identify the target bacteria in the presence of other microbes in food (Ohk, *et al.*, 2010; Kärkkäinen, *et al.*, 2011b). The capability of the aptamer pool 9 to detect *E. coli* K12 from a mixture of the bacterial cells was tested. FAM-labelled aptamers were incubated with a mixture of bacterial suspension (*E. coli* K12, *E. coli* B and *S. aureus)* and each suspension individually. In Figure 4.14, the fluorescence values of the samples are presented. In the diagram, it can be seen the highest fluorescence was detected from the mixture of these three bacterial suspensions (Mix) as expected. Minor fluorescence can be detected from *E. coli* B and *S. aureus* samples. The

positive control sample (*E. coli* K12) has the strongest fluorescence, as expected. The difference between the fluorescence values of the mixture sample and the positive control sample are small. These results indicated that the aptamers could detect *E. coli* K12 from a mixture of bacterial cells.

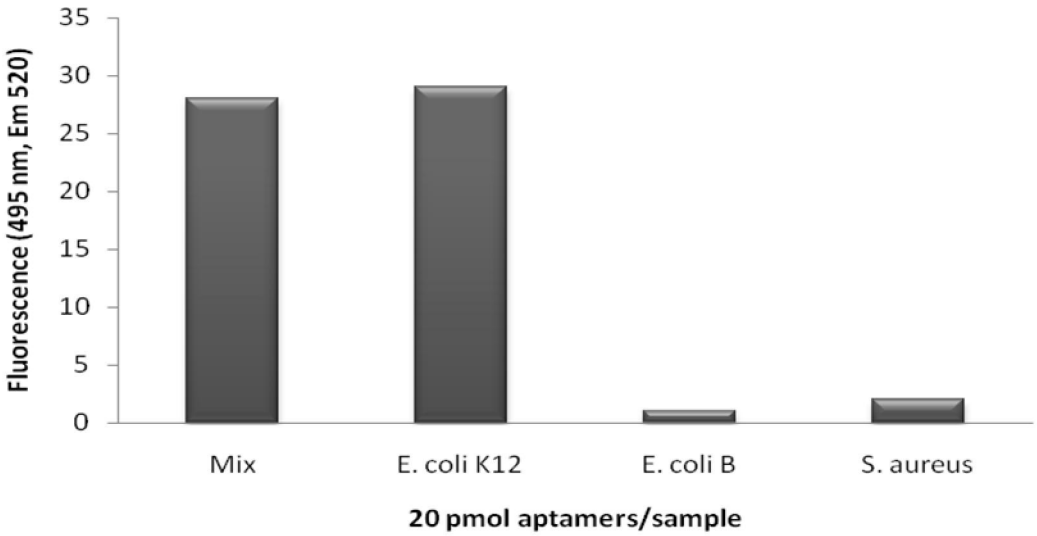


Figure 4.13 Detection of *E. coli* K12 from a mixture of bacterial cells. FAM-labelled aptamer pool nine was incubated with a mixture of *E. coli* K12, *E. coli* B and *S. aureus* (Mix) and with each strain separately. The fluorescence (495 nm, Em 520) was measured by a plate reader (n=1). Fluorescence has been corrected for background (Mix=23; *E. coli* K12=8; *E. coli* B=0; *S. aureus*=0).

4.3.4.3. Binding of the aptamers to *L. acidophilus*

The specificity test was performed with *L. acidophilus* and *E. coli* K12 was used as positive control. Aptamer pool 9 (20 pmol) was incubated with bacterial cells and the fluorescence of the samples was read by the plate reader. The results are presented in Figure 4.15. In the diagram, it can be seen that no fluorescence was detected in gram-positive *L. acidophilus* samples. The fluorescence of the positive control sample (*E. coli* K12) is significantly higher than the fluorescence in *L. acidophilus* samples. This result confirms the aptamer pool 9 is specifically binding to *E. coli* K12 and no binding to *L. acidophilus* can be detected.

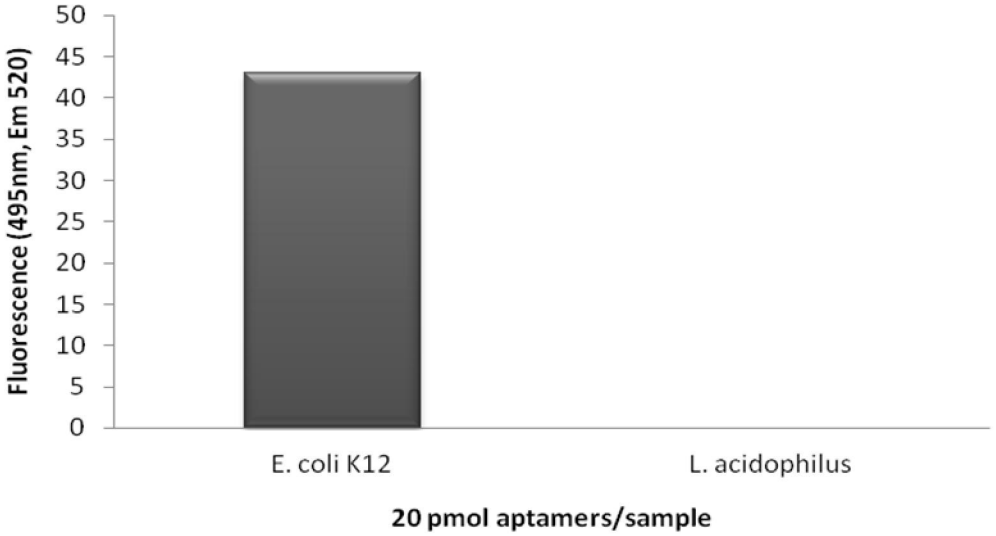


Figure 4.14 Specificity of *E. coli* K12 specific aptamer pool 9. FAM-labelled aptamers were incubated with *E. coli* K12 (positive control), *E. coli* B, *S. aureus* and *L. acidophilus*.The fluorescence (495 nm, Em 520) was measured by a plate reader (n=1). Fluorescence has been corrected for background (*E. coli* K12=6; *L. acidophilus*=25).

4.3.5. Fluorescent microspheres

Fluorescent labelled aptamers binding to smaller bacterial cells than *E. coli* K12 might be difficult to detect under the microscope. Also the FAM-label is small and therefore it might not always be visible in the fluorescence microscope images. Aptamers can be labelled with biotin (3.3.3) and by adding a larger fluorescent conjugate such as streptavidin (Terazono *et al.*, 2010) that can bind to biotin the microscopic detection may be possible. A concept of fluorescence microspheres technique was introduced to detect the aptamer binding.

Biotin labelled aptamer pool 9 was produced and the aptamers were incubated with *E. coli* K12 bacterial cells. The FluoSpheres were added and samples were incubated in order to let the microspheres bind to biotin label of the aptamers. The samples were visualised under the fluorescence microscope and the images were taken. Examples of the microscope images are shown in Figure 4.16. The bacterial cells can be seen in the images as well as the microspheres because the images were taken with a green fluorescence light and a visible light. Normal sized images are on left hand side and on right hand side are the zoomed images where the binding of the FluoSpheres can be seen. When comparing the negative control sample (-) to the sample (+) where the biotin aptamers have been added, it can be seen that some of

the microspheres have bound to the bacterial cells surface. This kind of binding cannot be seen in the control sample. The results indicated that the biotin-labelled aptamers and the fluorescence microspheres can be used in detection of aptamer binding. This method can possibly be used in detection of the aptamer binding to bacterial cells smaller than *E. coli* K12 but further optimisation of the method is needed.

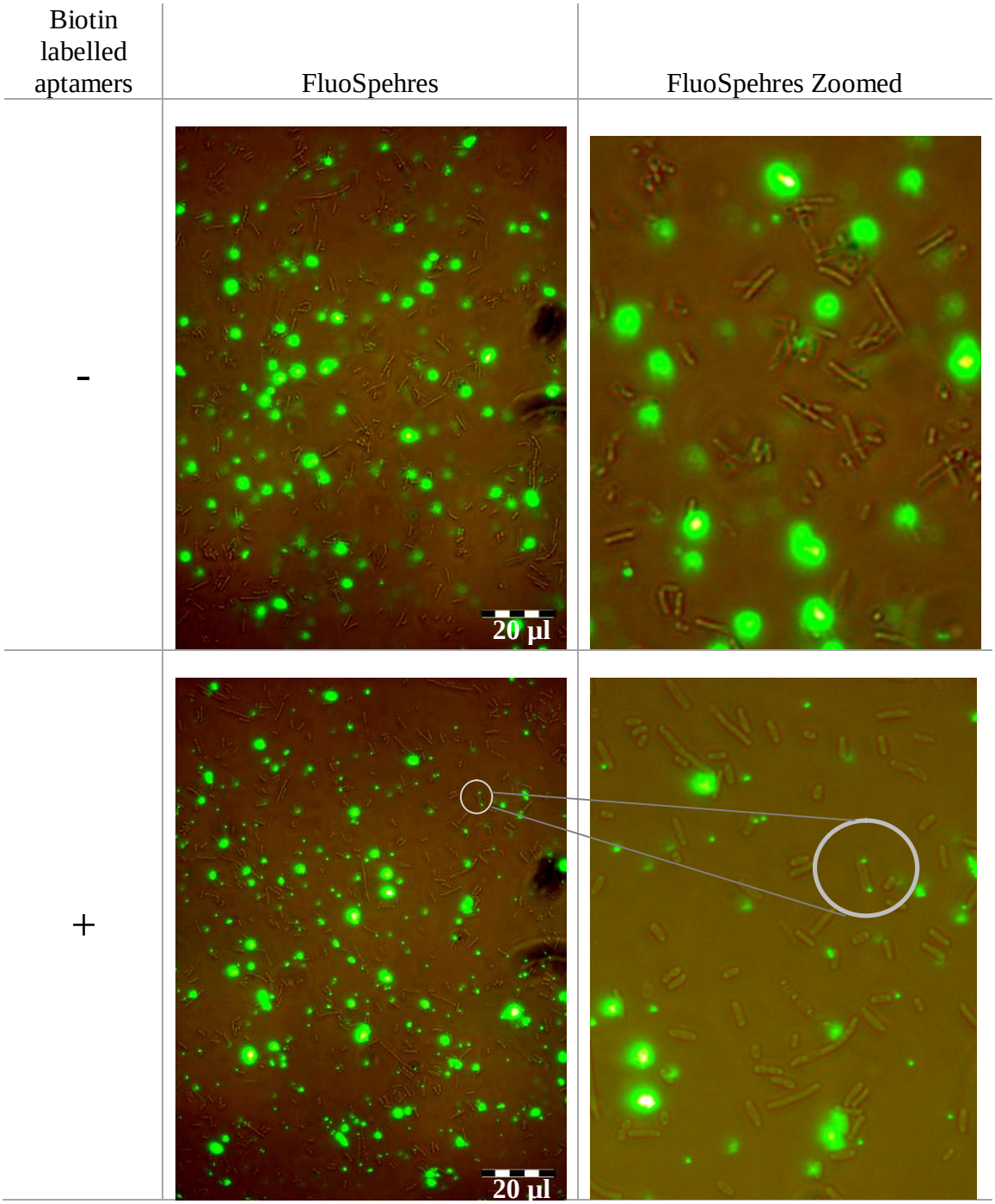


Figure 4.15 Biotin labelled aptamer pool 9 bound to *E. coli* K12. FluoSpheres were bound to biotin and the images were taken with a fluorescence microscope with 100× magnification. Biotin labelled aptamers incubated with *E. coli* K12 (+) and no aptamers added to the negative (-) control sample. Normal sized images are on left hand side and zoomed images on right hand side. Examples where FluoSpheres have bound to bacterial cell are circled.

4.3.6. Aptamer detection of live and dead *E. coli* K12 bacteria cells

An interesting application of aptamers would be to detect only live or dead bacterial cells. It may be important to be able to detect the live pathogenic bacteria cells that can cause food poisoning. The heat treatment of some food products, such as milk and cream cheese, might kill the bacteria but some microbes such as *Bacillus cereus* and *C. botulinum* (European Food Safety Authority, 2011) can produce toxins that remain in an active conformation and this way cause the poisoning. In this case, the detection of the toxin might be more important.

The Live/Dead *Bac*Light staining was performed in order to see if live or dead *E. coli* K12 bacterial cells could be detected with the aptamers. The FAM-labelled aptamer pool 9 was first incubated with *E. coli* K12 and the Live/Dead staining was performed. The FAM-labels were difficult to see because of the brighter colour from Live/Dead staining. In Figure 4.17 a fluorescence microscope image is shown. It can be seen that it is impossible to detect if the aptamers have bound to green, live bacterial cells. On red dead cells some green colour can be detected but this colour might also be derived from the Live/Dead staining kit where the colour has partly stained the bacterial cells.

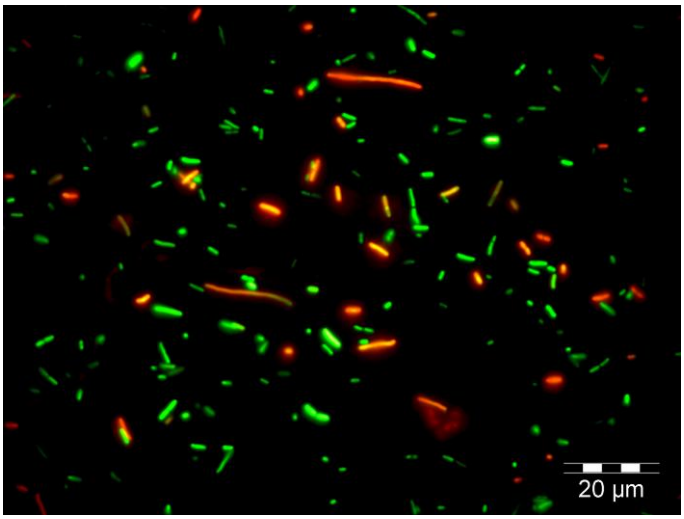


Figure 4.16 FAM-labelled aptamer pool 9 bound to *E. coli* K12 followed by Live/Dead *Bac*Light straining. The images were taken with a fluorescence microscope with 100× magnification.

In this study, biotin and its affinity to avidin and streptavidin was used as a basis (4.3.5). Biotin-labelled aptamer pool 9 was incubated with *E. coli* K12 bacterial cells

and the FluoSpheres were attached to them followed by Live/Dead staining of the cells. The microscope images are presented in Figure 4.18. It can be seen that some of the FluoSpheres have bound to bacterial cell surface on negative control samples (-) where biotin labelled aptamers have not been added. This indicates that the FluoSpheres might bind to bacterial cell surface. Not much binding can be detected on the sample (+) where the biotin labelled aptamers have bound to bacterial cells surface. Some of the molecules could have been washed off during the staining. Also, the bond strength between the aptamer and the bacterial cell is not known and it is possible that this bond breaks when the non-binding FluoSpheres are washed off. To select the aptamers which specifically bind to live or dead bacterial cells, the selection process may have to be started with a pool of aptamers which bind only molecules that are only present if the cells are alive or dead. The method described here has to be further optimised and it can then be used to screen the properties of the aptamers. It is still not clear if the LIVE/DEAD *Bac*Light staining affects the ability of the aptamers to bind to their target.

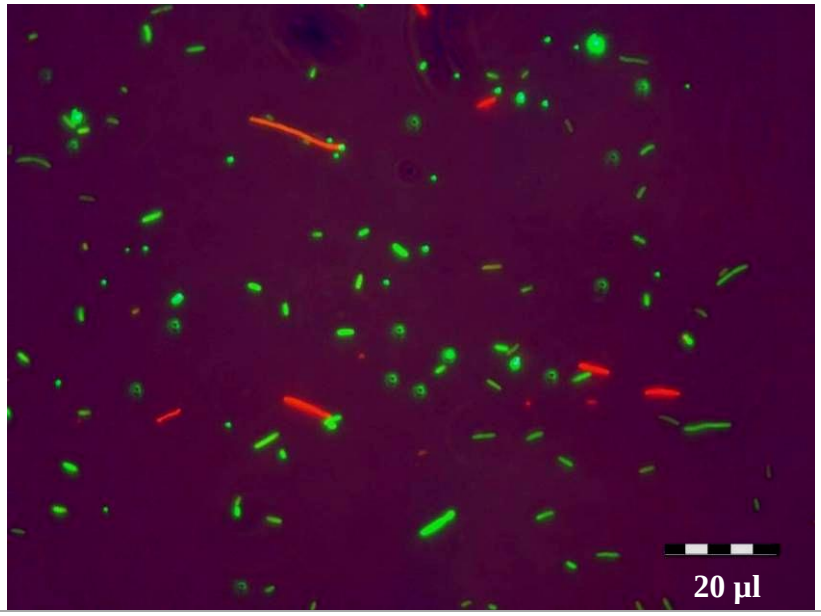
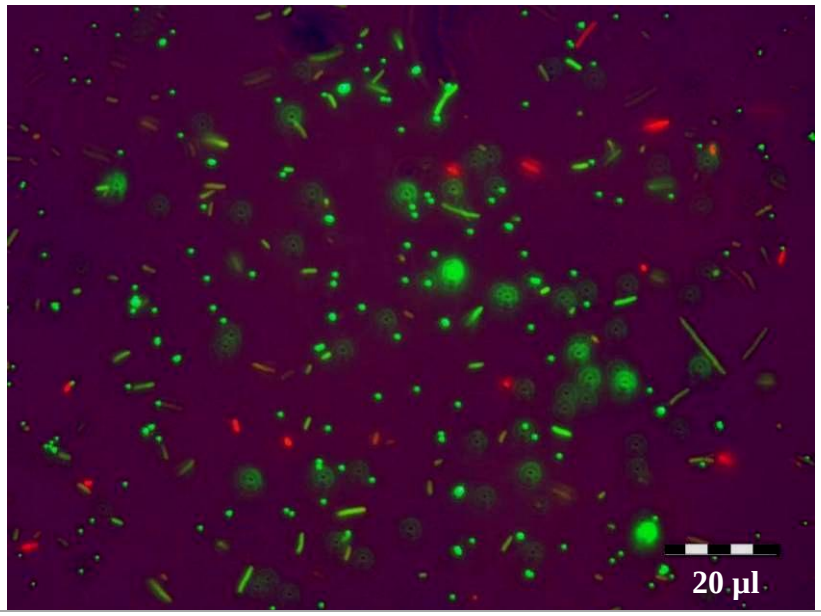
Biotin labelled *E. coli* K12 Aptamer pool 9	FluoSpheres and LIVE/DEAD *Bac*Light staining
-	
+	

Figure 4.17 Biotin labelled aptamer pool 9 bound to *E. coli* K12. FluoSpheres were bound to biotin followed by LIVE/DEAD *Bac*Light staining. The images were taken with a fluorescence microscope with 100× magnification. Biotin labelled aptamers were incubated with *E. coli* K12 (+) and no aptamers were added to the negative (-) control sample. Green colour indicates the cells are alive whilst red colour indicates the cells are dead.

4.4. CONCLUSIONS

Aptamers were selected to bind to *E. coli* K12 live bacterial cells. The binding was first detected with a method based on an enzymatic reaction. This method was time consuming. The easier and more reliable fluorescence-based method was developed and by using this method the binding properties of the aptamers were analysed.

The binding of the fluorescent-labelled aptamers was tested by comparing the fluorescence values of the samples. This method made the testing of the aptamer binding easier and allowed comparison of different samples with each other. The fluorescent labelled aptamer binding could also be visualised under a fluorescence microscope. However, this method cannot be used for statistical analysis, is time consuming and is not very reliable as the bacterial cells are not spread consistently over a microscope slide, but it gives interesting visual information about the aptamer binding. The results showed that the aptamers are binding to *E. coli* K12 bacterial cells. According to the microscope images, it was considered that the target bacterial cells were in dividing stage of the cell cycle.

It was found that the optimal binding time for the aptamers was 45 minutes and the binding was not significantly improved when the incubation time was increased. Three washes were enough to remove the non-binding aptamers. It was shown that most of the aptamers added to the reaction did not bind the bacteria and were washed off after the incubation. The specificity tests indicated that the aptamers were binding specifically to *E. coli* K12 bacterial cells and that the cells can also be detected when in a mixture of bacterial cells from other species. Not all bacterial cells from the other species were easy to see under a microscope as they might be smaller in size and move faster than *E. coli* K12. An alternative method was introduced where the same principles of visualising the samples under the fluorescence microscope were used. In this experiment, the aptamers were labelled with a biotin-label and fluorescent labelled microspheres were attached to them. The samples were then visualised under the microscope. The method tested in this study shows that the aptamer binding can be detected with the fluorescence beads. More information on the sensitivity of the aptamer pools could have been derived by testing the limit of the bound molecules that can be detected. This would have given

more information about the binding capacity and affinity of the selected aptamer pool.

The aptamer pool 9 specific to *E. coli* K12 would be tested to examine if the detection of *E. coli* K12 in a real food sample was possible, for example a yogurt. In order to synthesise the aptamers, the nucleotide sequence of the individual aptamers would have to be identified. It was decided that to achieve this, the aptamer pool selected to bind *E. coli* K12 would be cloned and the cloned plasmid would be sequenced. Once the individual aptamer sequences have been identified, the aptamers can be synthesised by a chemical synthesis with a FAM- or biotin-label and enabling analysis of the aptamer properties. Subsequently, the binding of these identified aptamer sequences will be tested against *E. coli* K12 and the specificity will be tested against different bacterial strains. The performance of the aptamer a food matrix will then be tested with the aptamer pool 9 as well as with the identified aptamers. More specific aptamers will be selected to bind to food poisoning bacteria.

CHAPTER 5 – Identification and sequencing of aptamers binding *Escherichia coli* K12

5.1. INTRODUCTION

Aptamers have been selected which demonstrated binding to live *E. coli* K12 bacterial cells. The binding was demonstrated by an enzymatic method (3.3.4) and by fluorescence-based methods (4.3.1). Although the selected aptamers bound specifically to *E. coli* K12 they were in the form of a mixture or pool of molecules with undefined nucleotide sequences. The identification of the individual aptamer sequences is an important part of the aptamer selection progress. When the aptamer sequences have been discovered the individual aptamers can be synthesised. The identification of individual aptamers within a pool can be achieved by cloning using competent bacterial cells (Ellington & Szostak, 1990).

In this study, the aptamer pool 9, which was specific to *E. coli* K12, was selected for cloning. The clones were analysed and the cloned plasmids were extracted and sequenced. From the plasmid vector sequence obtained by this procedure, the individual aptamer sequences would then be identified and the secondary structures established.

Since the synthesis of long nucleotide sequences is difficult and expensive, the nucleotide sequences of the aptamers are often reduced (James, 2007). A reduction below 60 nt is almost essential for efficient chemical synthesis and a size of 40 nt or below is desirable on grounds of cost of goods. It was therefore decided that a strategy would be adopted in which shortened aptamer sequences, derived from the initial aptamer secondary structures (Akitomi *et al.*, 2011), would be isolated and synthesised with a fluorescence label. Secondary structure analysis (Stoltenburg *et al.*, 2007) was done with a program that provides information of the possible aptamer configurations such as stems, loops and bulges. The previously developed fluorescence-based detection methods, based on fluorimetry and fluorescence microscopy, were again be used to detect the aptamer binding to the bacterial cells. As part of this study, the application of fluorescent aptamers to *E. coli* K12 cultures at different growth times was also performed to see whether the age of the bacterial

culture influence binding. Incubation of the individual fluorescent aptamers produced by the cloning process was also performed with *E. coli* B and *S. aureus* to further evaluate their specificity.

5.2. METHODS

5.2.1. Cloning of aptamers

The binding of aptamer pool 9 to *E. coli* K12 has been demonstrated (3.3.4; 4.3.1). So far, the aptamer pools with unidentified sequences have been produced by PCR. In order to synthesise the aptamers the individual sequences need to be determined. This was done through cloning of the aptamers (Ellington & Szostak, 1990).

5.2.1.1. Ligation and transformation

The *E. coli* K12 aptamer pool 9 was produced by PCR (3.3.2) and ligated into a pGEM-T easy vector using a method described in 2.3.12.1. It was decided to use 1:1 insert:vector ratio for the control insert DNA for pGEM-T easy vector while a optimisation of the ligation reaction for aptamers was recommended (Promega Technical Manual, 2009). The formula for molar ratio calculation was:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

In this study the insert:vector ratio was optimised for ratios 1:4, 1:2 and 1:1. The PCR product (insert) concentration was estimated to 0.8 ng/µl and the PCR product size was 100 bp (0.1 kb). The pGEM-T easy vector (50 ng) size is 3 kb. Therefore, the PCR product amounts for this reaction were 0.4 ng (1:4), 0.8 ng (1:2) and 1.7 ng (1:1). The ligation reaction components are listed in Table 5.1.

Table 5.1 Ligation reaction components for the samples and the controls.

	Insert:vector molar ratios				
Reaction component	**1:4**	**1:2**	**1:1**	**Background**	**Positive control**
Ligation buffer	5 µl	5 µl	5 µl	5 µl	5 µl
Vector pGEM-T Easy	1 µl	1 µl	1 µl	1 µl	1 µl
PCR product	0.5 µl	1 µl	2 µl	-	control insert 2 µl
Ligase	1 µl	1 µl	1 µl	1 µl	1 µl
H_2O	2.5 µl	2 µl	1 µl	3 µl	1 µl

The transformation of the ligation reaction (2 µl) into the competent cells was done as described in section 2.3.12.1. After the transformation the samples were incubated in SOC medium before plating 100 µl of suspension on LB plates with ampicillin/IPTG/X-gal. The colonies were counted from overnight grown selective plates (2.3.12.2). From the selective plates, eight positive white colonies were randomly selected and streaked on a new indicator plate. The plates were incubated overnight.

5.2.1.2. PCR analysis of the positive colonies

The colonies on the selective plates were analysed to see if the ligation and transformation had worked. The PCR analysis was performed for eight samples (Cl1-Cl8) from the overnight selective plates with PCR Supermix HiFi (2.3.12.3) and the aptamer primers PR1 and PR2. The templates used in this reaction were taken directly from the colonies. The initial denaturation of PCR cycle breaks down the bacterial cell walls releasing the plasmid DNA with an insert. The primers used are the same as the primers used for aptamer production. If the cloning of the insert (aptamer) and the transformation of the vector inside the bacterial cells have been successful, a 100 bp band should be visible on the agarose gel pictures. The PCR control (no template DNA) and a plasmid vector control (template 0.5 µl vector) samples were done. The plasmid DNA control is included to show if the plasmid and the primers produce non-specific amplification products. The PCR products were separated on an agarose gel.

5.2.1.3. EcoRI restriction analysis for plasmid vectors

The cloned eight colonies were also tested with a restriction analysis to see if the cloning has worked. Restriction analysis (2.3.12.5) was performed to confirm the results of PCR analysis. The colonies were inoculated into LB-media and the plasmid was purified from the overnight grown culture (2.3.12.4). EcoRI enzyme has digestion sites in the both sides of the insert and therefore was selected to digest the insert out from the plasmids (Cl1-Cl8). The digestion sites can be seen in the sequence of the pGEM-T Easy vector in Figure 2.4. The samples were separated on an agarose gel (2.3.2).

5.2.2. Binding of the cloned aptamers to *E. coli* K12

Two cloned colonies (Cl1 and Cl2) were randomly selected to test if they are binding to *E. coli* K12. The clones were produced by PCR (2.3.1) under the same conditions using the fluorescence FAM-primers as in the usual aptamer production. The template used in this reaction was a nested template for the cloned aptamers in dilution 1:60. The PCR products were separated on agarose gel followed by purification of the PCR product.

The overnight grown *E. coli* K12 culture was prepared as previously described (2.3.9.1). The bacterial suspension (100 µl) was incubated with 10 pmol, 20 pmol and 40 pmol of aptamers. No replicates were done in this experiment due to the small aptamer yield. The samples were washed and the fluorescence was measured by the plate reader (2.3.9.3).

5.2.3. Sequencing of cloned vectors and aptamer analysis

In order to determine out the aptamer sequences, the cloned aptamers were sequenced directly from the plasmid vectors. According to the results of the PCR analysis and the restriction analysis four positive samples were selected for the sequencing. Plasmid DNA samples Cl1-Cl4 (30 µl each) were sequenced (2.3.12.6). The aptamer sequences were identified from the vector sequence by identifying the primers binding sites of the primers PR1 and PR2 from the vector sequence. Between the primer sequences the aptamer sequence with 40 nucleotides long random sequence were found.

The aptamer binding to its target is dependent on its structure characterised by stems, loops, bulges and hairpins (Stoltenburg *et al.*, 2007). The possible secondary structures of the aptamer sequences were analysed as previously described (2.3.12.7) by using the UNAFold program. From the cloned and analysed sequences four aptamers (1AptK12, 2AptK12, 4AptK12 and 6AptK12) were selected and synthesised with a fluorescence FAM-label in its 5' end. It was decided to use four different sequences for the analyses and these four aptamers were selected because of their matching structure to the original 100 nucleotides secondary structure. Also the free energy, ΔG, was taken into account when selecting the aptamers to be synthesised and to be used for further analysis. Gibbs free energy was previously

described in Chapter 3 (3.3.1). In this study, the negative ΔG values given by the UNAFold program are representing the strength of the predicted aptamer structure. This means the smaller the ΔG, the stronger the structure.

5.2.4. *E. coli* K12 binding aptamers

5.2.4.1. Binding of the aptamers to *E. coli* K12

The binding properties of the sequenced aptamers were tested. Aptamers 1AptK12, 2AptK12, 4AptK12 and 6AptK12 were synthesised with 5' FAM-label and the binding to *E. coli* K12 was tested. The overnight grown *E. coli* K12 culture (2.3.9.1) was washed and incubated with the aptamers (10 pmol, 20 pmol, 50 pmol and 100 pmol) in triplicate. Due to the synthesis of single stranded molecules the denaturation of the aptamers is not necessary. The aptamers (10 µl) were added to 100 µl bacterial suspension in BB. The fluorescence of the samples was read by the plate reader (2.3.9.3). The values were compared by one way analysis of variance (ANOVA). To visualise the binding of the aptamers, 50 pmol samples were selected and visualised under the fluorescence microscope (2.3.9.2).

5.2.4.2. Specificity of the aptamers

The binding of the aptamers 1AptK12, 2AptK12, 4AptK12 and 6AptK12 to *E. coli* B and *S. aureus* was tested. *E. coli* K12 was used as a positive control. A mixture of the aptamers (50 pmol) was used in the binding reaction (2.3.9.1) in triplicate. The samples were incubated and after the washes the fluorescence was measured by the plate reader (2.3.9.3) and the samples were visualised under the fluorescence microscope (2.3.9.2). The fluorescence values were analysed using the statistic test ANOVA. The same protocol was then repeated with individual aptamers (20 pmol) in order to see the differences between different aptamers. Due to the problems with the plate reader the sensitivity had to be changed from 50 to 75 in order to read the plate.

5.2.4.3. Aptamer binding to *E. coli* K12 at different stages

The aptamer binding was tested with the *E. coli* K12 bacterial culture at different stages of the growth curve to see if the age of the culture affects the binding. The cultures were incubated for 4h, 8h, 16h, 20h and 24h. The mixture of the aptamers (20 pmol) 1AptK12, 2AptK12, 4AptK12 and 6AptK12 was incubated with the

cultures in triplicate. The aptamer concentration 20 pmol was used in this study, because previous experiments (5.2.4.1 and 5.2.4.2) have shown that 20 pmol is enough for the detection. The bacterial cells were prepared as previously described (2.3.9.1) except the 4h and 8h samples where a double volume of bacterial suspension was washed and resuspended into 500 µl BB. This was done because of the small number of the bacterial cells in 4h and 8h samples. The samples were visualised under the fluorescence microscope (2.3.9.2).

5.3. RESULTS AND DISCUSSION

5.3.1. Cloning of aptamers

5.3.1.1. Ligation and transformation

Aptamer pool 9 with specificity for *E. coli* K12 was developed through selection process (3.3.2) and the binding of the aptamers against their target was confirmed by the enzymatic method (3.3.4) and a method based on fluorescence (4.3.1). As a part of the well established aptamers selection process (Tuerk & Gold, 1990; Ellington & Szostak, 1990), aptamer pools that contain a mixture of unidentified aptamer sequences have to be cloned in order to determine the individual aptamer sequences.

Aptamer pool 9 was amplified by PCR and cloned with pGEM-T Easy vector and JM109 competent cells. The colonies on indicator plates were counted and the number of positive (white) and negative (blue) colonies is presented in Table 5.2. The number of colonies on the plates was small. The positive control sample was spread on a selective plate, where the ampicillin used was not fresh and therefore might have lost its activity. On a normal selective plate, the positive control samples express the colonies if the transformation has been successful. Due to the inactive ampicillin the plates were full of colonies. When the ampicillin is normally used, the positive colonies are only growing on the plates due the ampicillin resistance of the bacteria. Even though the positive control sample was full of colonies, further analyses of the actual samples were performed. Eight white colonies were randomly selected from the plate and streaked on new selective plates. After an overnight incubation on selective plates, five colonies (Cl3, Cl4, Cl6, Cl7 and Cl8) out of eight expressed the blue colour while three remained white (Cl1, Cl2 and Cl5). The blue colour may be expressed even if the insert has been ligated into the vector. In a

normal situation where the insert has successfully been ligated the reading frame for the *lac*Z gene has been interrupted. This means that the enzyme β-galactosidase is not expressed and therefore the blue coloured colonies are not formed on the selective plates containing the X-Gal and IPTG (Campbell & Farrell, 2009). If positive blue colonies are formed the PCR products have been cloned in-frame with the *lac*Z gene or it may be caused by the mutations (deletions or point mutations) (Promega Technical manual, 2009).

Table 5.2 Number of colonies on indicator plates. The colonies were counted after overnight incubation.

Insert:Vector molar ratio	Blue (negative)	White (positive)	Total
1:4	3	2	5
1:2	20	21	41
1:1	1	2	3
Background	1	2	3
Positive control	∞	∞	∞

5.3.1.2. PCR analysis of the positive colonies

The colonies from the selective plates were analysed to see if the insertion of the aptamers into the vector has been successful. The PCR analysis was performed for the cloned colonies as suggested by TOPO TA Cloning® Kit Manual (Invitrogen, 2006). The aptamer primers PR1 and PR2 were used in this experiment. Therefore, only the colonies with the aptamer sequence and the primer binding sites are amplified in PCR resulting in a 100 bp products. If 100 bp long bands can be seen on a gel the aptamer cloning has been successful.

The agarose gels of the PCR amplification products are presented in Figure 5.1. It can be seen that the samples Cl1, Cl2, Cl3 and Cl4 have a 100 bp band and a very faint band can be seen in sample Cl6. This result shows that the cloning of the aptamers has been successful and the cloned insert has been amplified. No PCR product can be seen in sample Cl5, Cl7 and Cl8. The PCR control sample (0) is clear and no amplification products can be seen, as expected. The faint band that can be seen on lane 0 is the primer dimer. No PCR products can be seen in the control sample (c), as expected. This result indicates the insert vector itself does not offer binding sites for the primers used in this experiment. Even the colonies on the second

indicator plates were expressing blue colour (Cl3, Cl4, Cl6, Cl7, Cl8), the PCR analysis gave a positive result for these samples. Also white colonies (Cl1, Cl2, Cl5) were stated to be positive, but according to the PCR analysis, samples Cl1 and Cl2 were positive and Cl5 negative. The results presented here suggest the colour selection is not appropriate for the positive colony selection because also the blue colonies might have the insert in their vector plasmid. In a typical situation the insert inactivates the α-peptide that codes an enzyme, β-galactosidase, resulting in the white colonies on indicator plates. In this study, the β-galactosidase production was not inhibited and therefore positive blue colonies were formed. Similar findings have previously been made by Bruno *et al.* (2009).

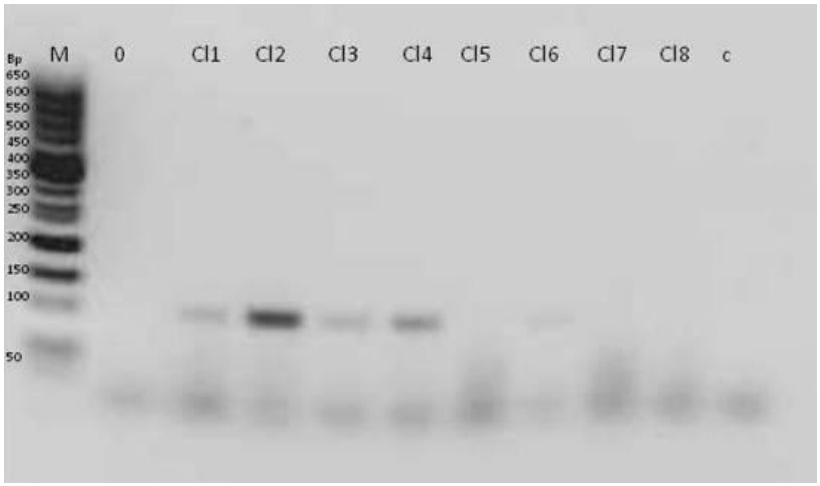


Figure 5.1 Agarose gel picture of the PCR Spermix HiFi analysis for positive colonies. Lane M on the gel contains PCR MiniSizer 50bp DNA Ladder and in lane 0 is a PCR control sample. In lane Cl1-Cl8 are the cloned colonies. Sample c is the plasmid control sample.

5.3.1.3. EcoRI restriction analysis for plasmid vectors

Cloned colonies were analysed with the PCR analysis to see if the cloning has been successful. The same samples were then analysed with a restriction analysis as suggested by the manual (Promega Technical manual, 2009) to get a confirmation for the results from the PCR analysis. The plasmids were extracted from overnight grown bacterial cell suspensions. The size of the whole pGEM-T Easy vector is 3015 bp and the insert aptamers are 100 bp. The total size of the cloned plasmid is 3115 bp. The EcoRI cut the insert out from the vector close to the insert adding 13 bases

more to the actual insert. Therefore the expected insert size to be seen on a gel is 113 bp and for the vector 3002 bp.

The agarose gel for the separated samples is presented in Figure 5.2. The pGEM-T Easy vector bands (Plasmid DNA) and the digested inserts (aptamers) are in lanes Cl1 – Cl8. It can be seen that the plasmid purification has been successful in all of the samples. The large plasmid DNA has not moved far on the gel and the range of the molecular weight marker does not reach to it. Also the intensity of the plasmid DNA bands is high when compared to the intensity of the insert bands. The lighting of the picture has been adjusted in order to get the faint insert band visible. A clearer image could have been achieved by running two gels, one for the plasmids and another for the inserts. The insert band can be seen in all of the samples except Cl1 and the band for Cl2 is very faint and can hardly be detected. It was expected that a 113 bp band would be seen in sample Cl1 when comparing the results for the PCR analysis (Figure 5.1) where Cl1 was positive. It is possible that the restriction analysis did not work for this sample, or the DNA concentration was too low to be detected from the gel.

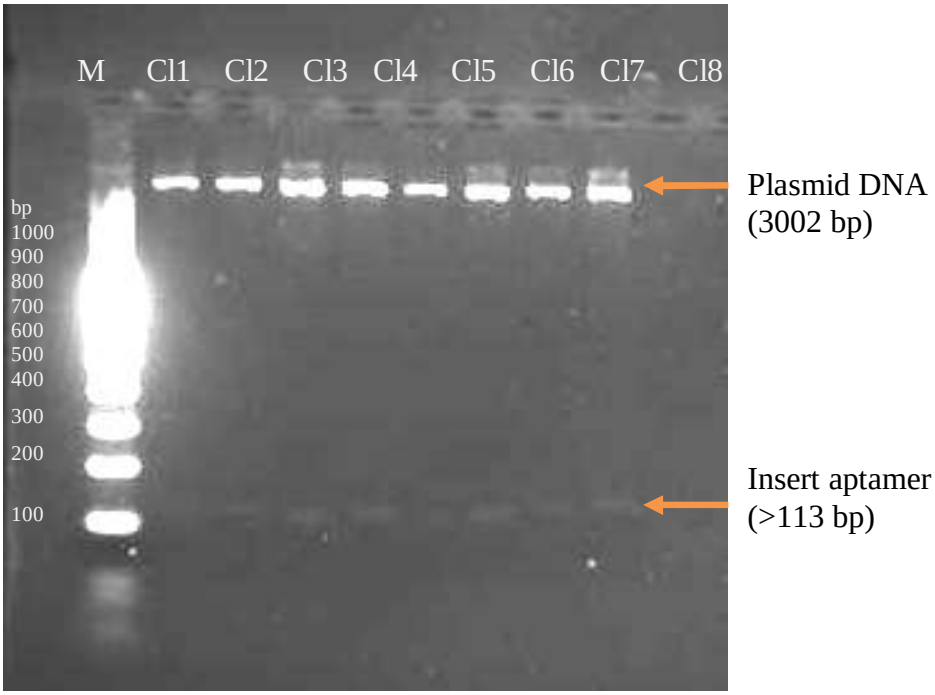


Figure 5.2 Agarose gel picture of the restriction (EcoRI) products. Lane M on the gel contains PCR Sizer 100bp DNA Ladder. Restriction products for cloned plasmids are in lanes Cl1- Cl8.

5.3.2. Binding of the cloned aptamers to *E. coli* K12

The binding of two aptamer clones with still unknown nucleotide sequences to *E. coli* K12 was demonstrated to see if these aptamers are still binding their target. The aptamers were produced using the usual aptamer production method (2.3.6). Two different cloned aptamers were used as a template. The agarose gel of the PCR products is presented in Figure 5.3. Cl1 and Cl2 cloned aptamers were produced with FAM-labels. The PCR products are on lanes Cl1 and Cl2. All samples have a strong 100 bp band. In lane 0 is the PCR control sample where only primers were amplified without a template. No amplification products can be seen in this lane as expected. The faint band (< 50 bp) in this lane is the primer dimer. The PCR products were purified with spin columns.

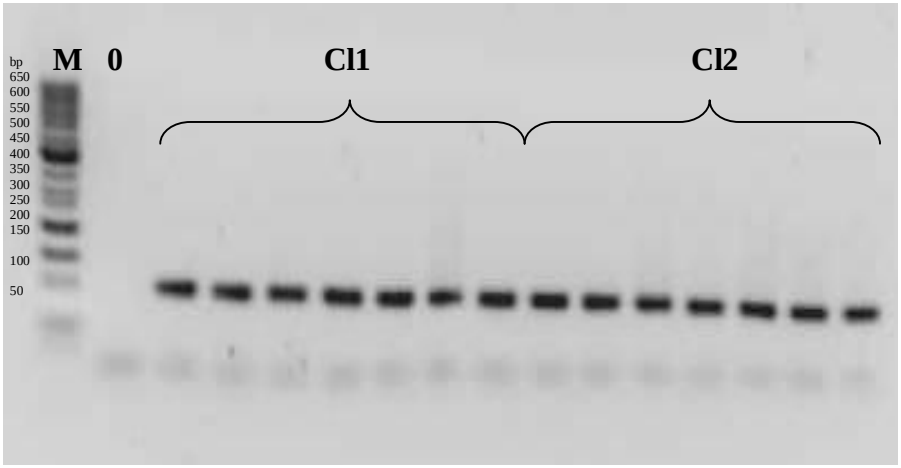


Figure 5.3 Agarose gel picture for FAM-labelled cloned aptamers Cl1 and Cl2. Lane M on the gel contains PCR MiniSizer 50bp DNA Ladder and in lane 0 is a PCR control sample (no template DNA added).

The purified FAM-labelled aptamers were incubated with *E. coli* K12 using aptamers in three different concentrations (10 pmol, 20 pmol and 40 pmol). No replicates were used (n=1) because of the small aptamer yield and the results were only presenting if the binding of the cloned aptamers can be detected. The fluorescence of the samples was measured using a plate reader. The fluorescence values are shown in Figure 5.4 where one can see that the fluorescence is higher when more aptamers have been added. This demonstrated that the cloned aptamers could bind to *E. coli* K12 bacterial cells. It is possible that these two different samples would have produced the curves closer to each other if the replicates had been performed.

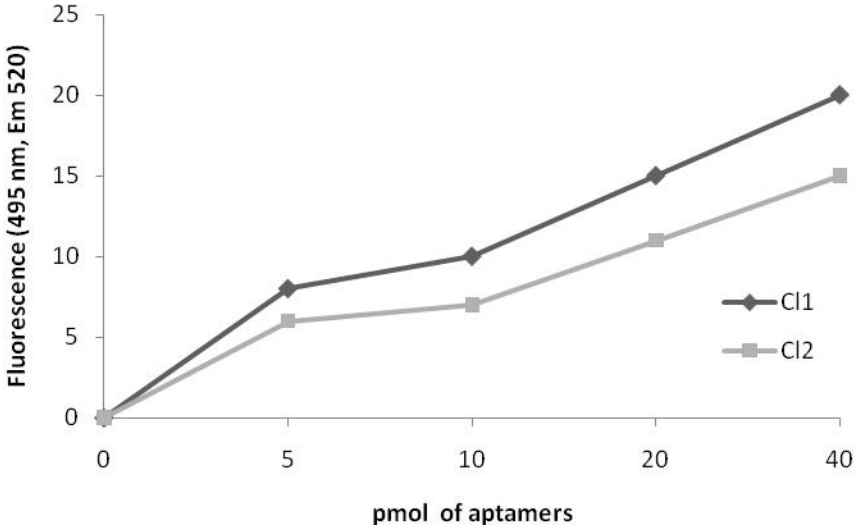


Figure 5.4 FAM-labelled cloned aptamers Cl1 and Cl2 binding to *E. coli* K12 cells. The fluorescence (495 nm, Em 520) was measured by a plate reader (n=1). Fluorescence has been corrected for background (4.5).

More clones can be randomly selected from the indicator plates and the binding tested in order to select more aptamers with the highest binding properties for the sequencing. Another possibility to select the clones for sequencing is to use a technique based on the aptamer structures (Gening *et al.,* 2001). After the aptamer selection the diversity of the different molecules decreases and the SELEX usually leads to separate groups of identical sequences that account for over 30% of the pool. To identify these pools a method based on single strand conformational polymorphism (SSCP) can be used. The ssDNA aptamers can be separated on non-denaturing polyacrylamide gel, because the migration of the molecules changes on the gel depending on their 3D structure. Aptamers with identical mobility are likely to have the same sequence. The majority of the clones having the same sequence or the same migration on the gel are the aptamers with the best binding properties. This technique can be used for initial characterisation of individual aptamer clones produced at the final stage of the selection. In this study the aptamers have been selected to bind to whole bacterial cells and it is possible that the enrichment of the sequences is not similar than when the aptamers have been selected to bind to a single molecule.

5.3.3. Sequencing of cloned vectors and aptamer analysis

The aptamer pool 9 specific to *E. coli* K12 had demonstrated to be binding to its target (4.3.1). The aptamers in this pool were still unidentified and to obtain the information for the individual aptamers, the cloned aptamers need to be sequenced. Four positive colonies were selected and the plasmid vectors were extracted. The aptamer sequences were determined by sequencing these vectors. By comparing the sequences to the aptamer primer sequences, PR1 and PR2, the aptamer sequences were identified from the vector.

Nucleotide sequences of the aptamers are presented in the Table 5.3. The length of the original DNA library, where the aptamers were selected from, was 100 nt (27 nt and 33 nt long primer binding sites and 40 nt random sequence). The size of the molecules in the DNA library was previously discussed when the DNA library was created (3.3.1). It was considered that the PCR products were 100 bp long as expected even the bands on the gel seemed bigger than 100 bp. The cloning results confirm that the size of the molecules in the original DNA library that was created by PCR is 100 bp. In Table 5.3 the 40 nt random sequence is underlined and the primer binding sites are on both sides of the sequences. The T7 and Sp6r sequences of each sample are complement strands to each other as they are sequenced from the same vector in different directions. Some of the aptamer sequences start with the same sequence as the primer PR1 and some of the sequences start with a sequence of primer PR2. This is due to the unknown direction when the insert is ligated into the vector. The first sample was 99 nt long (Cl1-AptK12) and can be caused by a deletion of one nucleotide during the sequencing. The rest of the samples were 100 nt sequences (Cl2-AptK12, Cl3-AptK12 and Cl4AptK12), as expected. The 99 nt long Cl1-AptK12 can be seen on the agarose gel (Figure 5.1) of the PCR analysis. One nucleotide differences cannot be detected on an agarose gel. Although on the gel picture, it looks like the band (Cl1) is slightly bigger than the band next to it on lane (Cl2). On the gel image of the restriction analysis samples in Figure 5.2 no bands can be seen in Cl1 sample. These results may indicate that this Cl1 is different from the others.

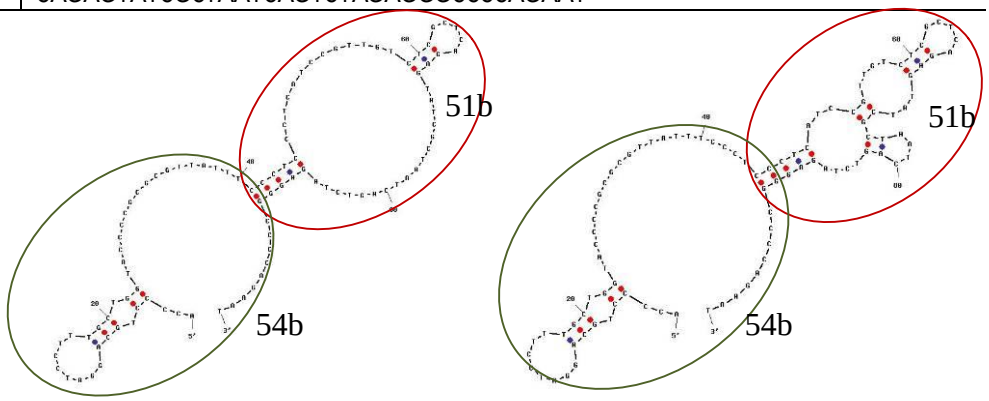
Table 5.3 A list of cloned *E. coli* K12 binding aptamers and their sequences.

Sample	Size/nt	Primer	Sequence
Cl1-AptK12	99	T7	ACCCCTGCAGGATCCTTTGCTGGTACC*AGAGCGGAGGGGCGTGAGGGGAGGGAGCTATGAGTGGAA*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
	99	Sp6r	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*TTCCACTCATAGCTCCCTCCCCTCACGCCCCTCCGCTCT*GGTACCAGCAAAGGATCCTGCAGGGGT
Cl2-AptK12	100	T7	ACCCCTGCAGGATCCTTTGCTGGTACC*CCGCGCGTTATTTCCCTCCCTCATCCGTTGTCTCGCTCAG*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
	100	Sp6r	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*CTGAGCGAGACAACGGATGAGGGAGGGAAATAACGCGCGG*GGTACCAGCAAAGGATCCTGCAGGGGT
Cl3-AptK12	100	T7	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*ACTTAACCTGCACACCCTCCCAAATCACTGCTCCCCCCCC*GGTACCAGCAAAGGATCCTGCAGGGGT
	100	Sp6r	ACCCCTGCAGGATCCTTTGCTGGTACC*GGGGGGGGAGCAGTGATTTGGGAGGGTGTGCAGGTTAAGT*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
Cl4-AptK12	100	T7	ACCCCTGCAGGATCCTTTGCTGGTACC*GCGTTATGGGAAAATCAGGAGAGAGGGGGAGGGAGAAAGT*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
	100	Sp6r	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*ACTTTCTCCCTCCCCCTCTCTCCTGATTTTCCCATAACGC*GGTACCAGCAAAGGATCCTGCAGGGGT

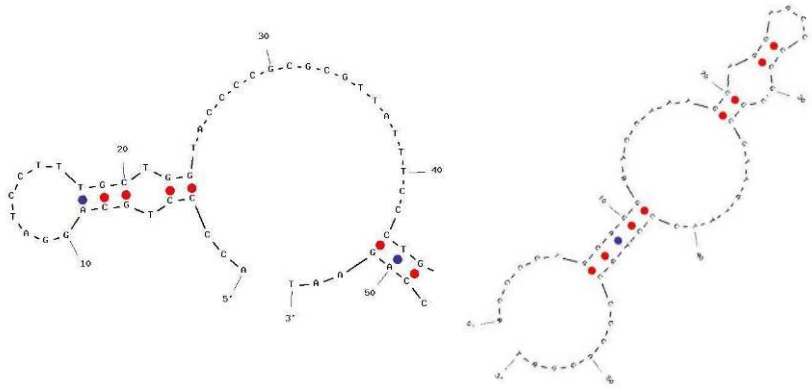
For further analysis, only the 100 nt aptamers (Cl2-AptK12, Cl3-AptK12 and Cl4-AptK12) were selected. Cl1-AptK12 was left out because the sequence did not meet the criteria of 100 nt and may not work properly. In order to synthesise the aptamers the number of nucleotides was reduced (James, 2000). The aptamers were analysed using UNAFold program that gives aptamers secondary structures most likely to be formed in the binding buffer conditions. The predicted structures are presented in Figure 5.5 (Cl2-AptK12), Figure 5.6 (Cl3-AptK12) and Figure 5.7 (Cl4-AptK12). Two structures for 100 nt sequences are presented on the first line and the aptamer sequences that were cut off from these 100 nt sequences are presented below. The reduced length sequences are marked by circles in the pictures and the isolated sequences and the secondary structures are presented for each sample. By comparing the secondary structures formed as well as the energy needed to form the structure (ΔG), four aptamers were selected and synthesised with FAM-labels. The ΔG was calculated by the UNAFold and it is defined as a change in Gibbs energy when the

system undergoes a thermodynamical change (Campbell & Farrell, 2009). The sequences and the details of the synthesised aptamers are listed in Table 5.4. Only four aptamers were synthesised and two aptamers were left out. Aptamer 3AptK12 was not synthesised because the first secondary structure is not matching the original 100 nt structure. Aptamer 5AptK12 was not selected because of the very weak secondary structure (ΔG= -1.55).

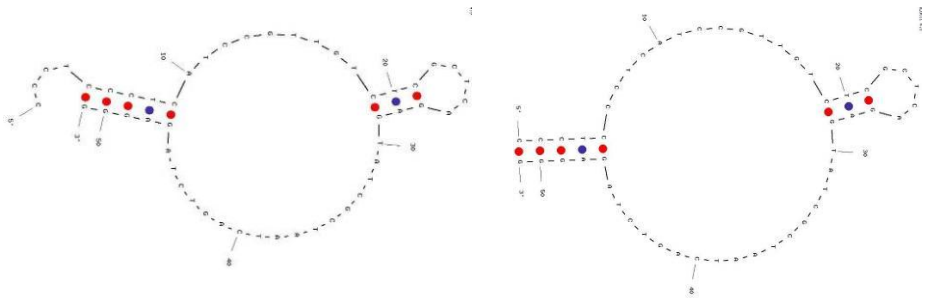
Cl2-AptK12	ACCCCTGCAGGATCCTTTGCTGGTACCCCGCGCGTTATTTCCCTCCCTCATCCGTTGTCTCGCT CAGAGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT



ΔG/Tm(°C)	-6.47/43	-5.59/36
1AptK12 54 nt	ACCCCTGCAGGATCCTTTGCTGGTACCCCGCGCGTTATTTCCCT*GCCCCAGAAT	



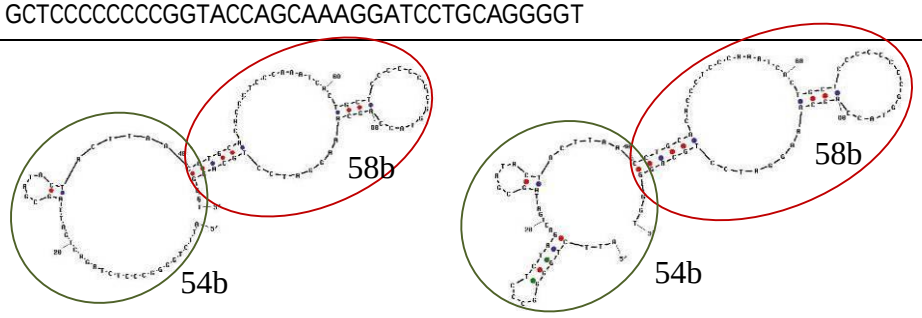
ΔG/Tm(°C)	-4.54/45.5	-3.56/39.5
2AptK12 51 nt	*CCCTCCCTCATCCGTTGTCTCGCTCAGAGTATCGCTAATCAGTCTAGAGGG*	



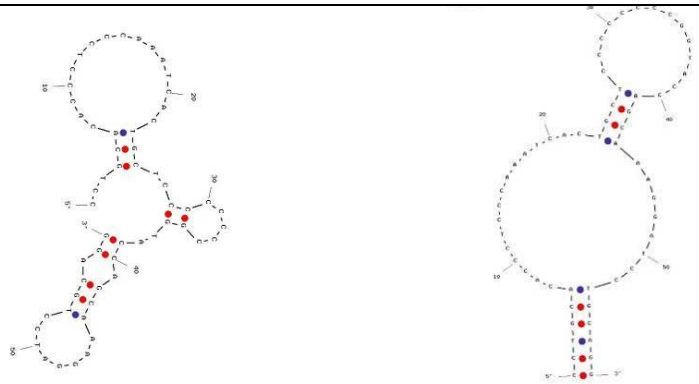
ΔG/Tm(°C)	-2.71/37.7	-2.16/39.8

Figure 5.5 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25 °C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *E. coli* K12 binding nucleotide sequence (Cl2-AptK12). Aptamers 1AptK12 and 2AptK12 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Dots represent the base-pair interactions.

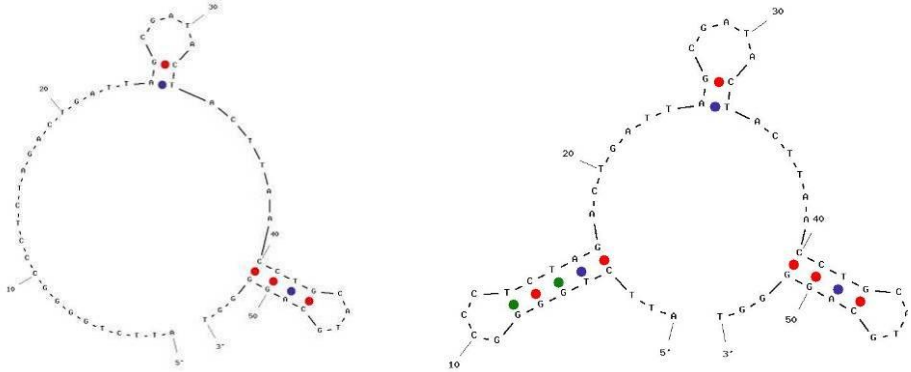
Cl3-AptK12	ATTCTGGGGCCCTCTAGACTGATTAGCGATACTACTTAACCTGCACACCCTCCCAAATCACT GCTCCCCCCCCGGTACCAGCAAAGGATCCTGCAGGGGT



ΔG/Tm(°C)	-4.97/41.1	-4.84/38
3AptK12 58 nt	*CCTGCACACCCTCCCAAATCACTGCTCCCCCCCCGGTACCAGCAAAGGATCCTGCAGG*	



ΔG/Tm(°C)	-3.05/36.5	-3.02/38.3
4AptK12 54 nt	ATTCTGGGGCCCTCTAGACTGATTAGCGATACTACTTAACCTGCA*TGCAGGGGT	



ΔG/Tm(°C)	-4.63/51.1	-4.5/43.5

Figure 5.6 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25 °C (NaCl 100 mM, MgCl 1 mM).The two strongest secondary structures for *E. coli* K12 binding nucleotide sequence (Cl3-AptK12). Aptamers 3AptK12 and 4AptK12 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Dots represent the base-pair interactions.

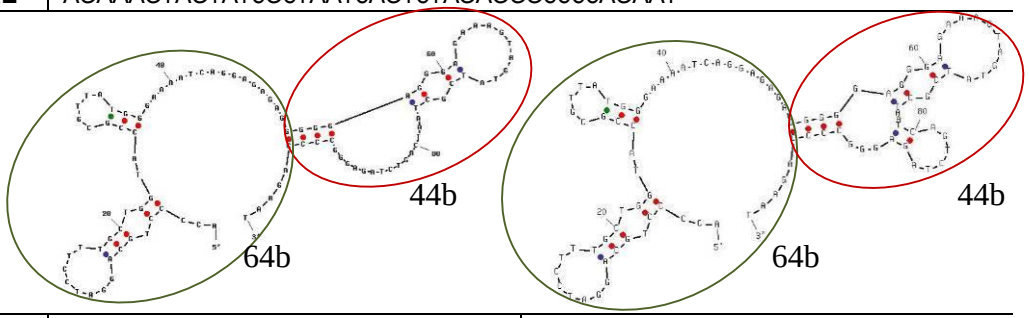
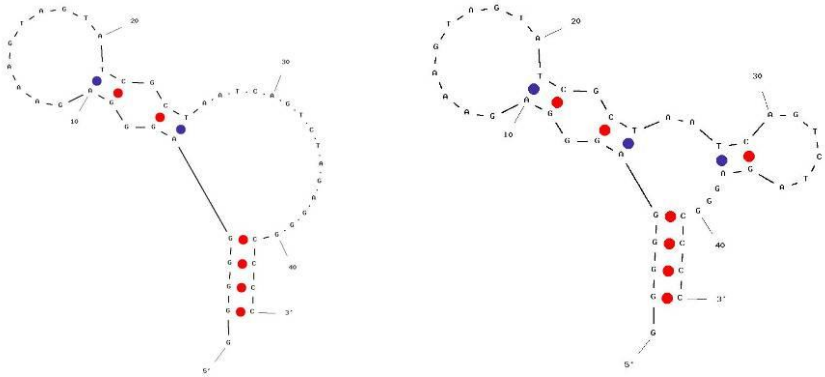
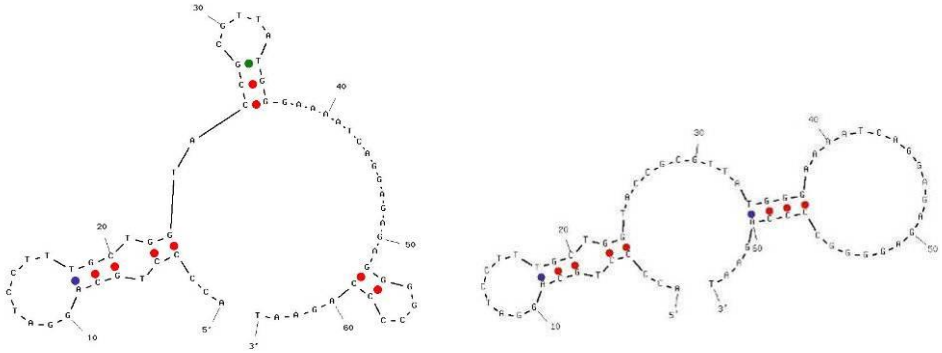
Cl4-AptK12	ACCCCTGCAGGATCCTTTGCTGGTACCGCGTTATGGGAAAATCAGGAGAGAGGGGGAGGG AGAAAGTAGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT	
		
ΔG/Tm(°C)	-4.97/41.1	-4.84/38
5AptK12 44 nt	*GGGGGAGGGAGAAAGTAGTATCGCTAATCAGTCTAGAGGGCCCC*	
		
ΔG/Tm(°C)	-1.55/33.6	-1.14/29.5
6AptK12 64 nt	ACCCCTGCAGGATCCTTTGCTGGTACCGCGTTATGGGAAAATCAGGAGAGAGGGG*CCCCA GAAT	
		
ΔG/Tm(°C)	-5.78/44.4	-5.38/46.1

Figure 5.7 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25 °C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *E. coli* K12 binding nucleotide sequence (Cl4-AptK12). Aptamers 5AptK12 and 6AptK12 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Dots represent the base-pair interactions.

Table 5.4 Sequences and ΔG values for the synthesised FAM-labelled aptamers. The ΔG values show the strength of the predicted aptamer secondary structure where the smallest number is the strongest structure.

Aptamer	nt	Sequence	ΔG
1AptK12	54	5'FAM-ACCCCTGCAGGATCCTTTGCTGGTACCCCGCGCGTTATTTCCCTGCCCCAGAAT-3'	-4.54
2AptK12	51	5' FAM-CCCTCCCTCATCCGTTGTCTCGCTCAGAGTATCGCTAATCAGTCTAGAGGG-3'	-2.71
4AptK12	54	5' FAM-ATTCTGGGGCCCTCTAGACTGATTAGCGATACTACTTAACCTGCATGCAGGGGT-3'	-4.63
6AptK12	64	5' FAM-ACCCCTGCAGGATCCTTTGCTGGTACCGCGTTATGGGAAAATCAGGAGAGAGGGGCCCCAGAAT-3'	-5.78

5.3.4. *E. coli* K12 binding aptamers

5.3.4.1. Binding of the aptamers to *E. coli* K12

Before the aptamers were cloned and sequenced the binding of the aptamer pool against its target *E. coli* K12 was shown (3.3.4; 4.3.1). The binding of the aptamer sequences to their target was tested. The aptamers (Table 5.4) were synthesised with a FAM-label and incubated (10, 20, 50 and 100 pmol) with *E. coli* K12 in triplicate. After the washes the fluorescence was measured using a plate reader and the fluorescence values are presented in Figure 5.8. It can be seen that the values are significantly higher when the aptamers have been added (1AptK12: F=40.54, p=3.77×10^{-6}, 2AptK12: F=28.99, p=1.77×10^{-5}, 4AptK12: F=97.4, p=5.77×10^{-8}, 6AptK12: F=52.46, p=1.12×10^{-6}). The results show that the highest fluorescence value measured was for 4AptK12 and the second highest for 6AptK12 followed by 1AptK12 and the lowest was measured for 2AptK12 when 50 pmol and 100 pmol aptamers were added. The results indicate that the aptamer 4AptK12 has the strongest binding to *E. coli* K12. There is no significant difference between the four different aptamers when 10 pmol aptamers were added (F=2.4, p=0.14) but the rest of the samples 20 pmol (F=6.39, p=0.02), 50 pmol (F=14.49, p=1.3×10^{-3}) and 100 pmol (F=31.5, p=8.83×10^{-5}) are significantly different. The difference between the samples is bigger when more aptamers have been added. When looking at the fluorescence values of individual aptamers, it can be seen that the fluorescence is not

much higher when 100 pmol of aptamers were added when compared to the samples where 50 pmol aptamers were added. The fluorescence values for 100 pmol samples are significantly higher than the 50 pmol samples of the 1AptK12 (F=17.02, p=0.01), 4AptK12 (F=14.39, p=0.02) and 6AptK12 (F=23.3, p=8.5×10^{-3}) but not in sample 2AptK12 (F=2.78, p=0.17) when 100 pmol aptamers were added. These results indicate that if more than 100 pmol aptamers have been added to the samples, the fluorescence might not increase much.

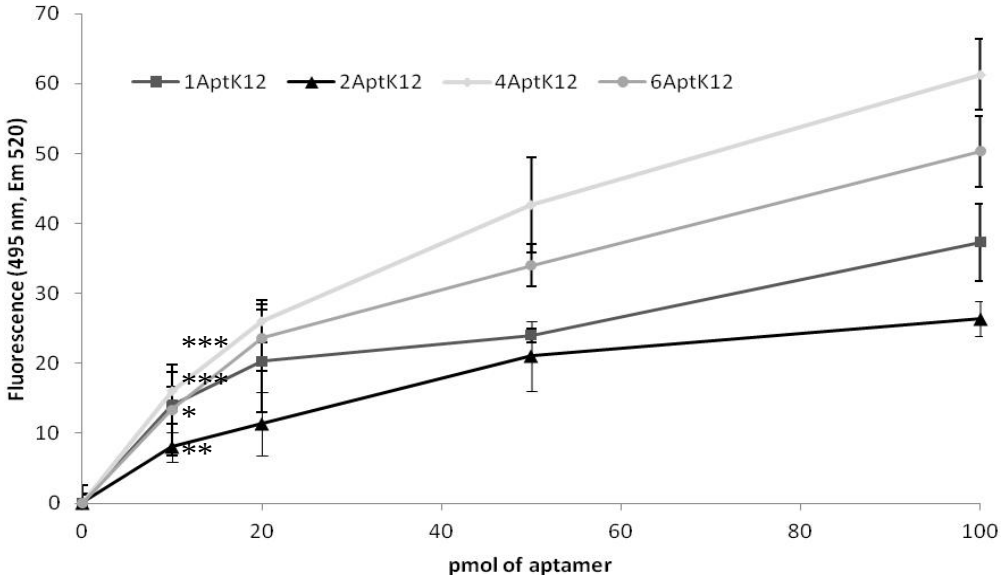


Figure 5.8 FAM-labelled aptamers binding to *E. coli* K12 cells. The fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (14.1). *Significant (P≤0.05), **Highly significant (P<0.01), ***Very highly significant (P<0.001).

Samples were visualised under the microscope with a green fluorescent and a visible light. The results for the 50 pmol samples are shown in Figure 5.9, where the fluorescent microscope images are on the left hand side and the visible light images are on the right hand side. The bacterial cells with fluorescent-labelled aptamers bound to them can be seen as bright green dots. It is noticeable the sample 4AptK12 has the brightest green dots. When comparing the result for the fluorimetry (Figure 5.8) it can be seen that the highest fluorescence was measured for this same sample. The second brightest dots are in sample 6AptK12. Also, the fluorimetry measurement gave the second strongest fluorescence for this sample. The lowest fluorescence in fluorimetry measurement was for the samples 1AptK12 and

2AptK12. This can also be seen in microscope images (Figure 5.9). Interestingly, aptamer 2AptK12 has the highest ΔG value (-2.71) (Table 5.4) and therefore the most unstable structure of these four aptamers. In the visible light image (right hand side), it can be seen that for the 2AptK12 the number of bacteria on the microscope slide is smaller than the other samples. This can also be the reason for the lower fluorescence. The strongest structure was 6AptK12 and the second strongest 4AptK12 according to ΔG values (Table 5.4). Also the strongest fluorescence was measured (Figure 5.8) and the brightest fluorescence dots can be seen (Figure 5.9) in these two samples. Similar result was obtained by Hamula *et al.* (2008) where the aptamer having the most energetically favourable secondary structure had the best binding properties. By comparing the visible light images and the fluorescence images, it can be seen that only some of the bacterial cells have been detected with the FAM-aptamers. This has also been demonstrated previously (4.3.1.2).

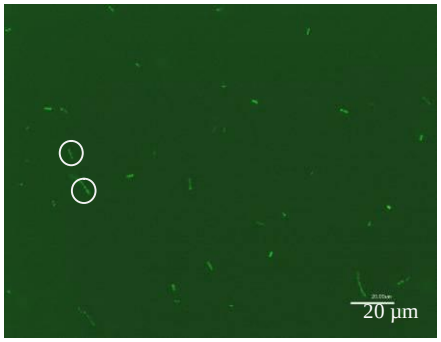
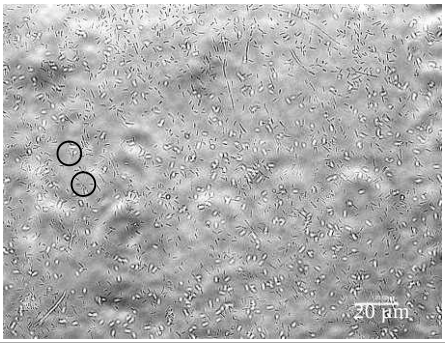
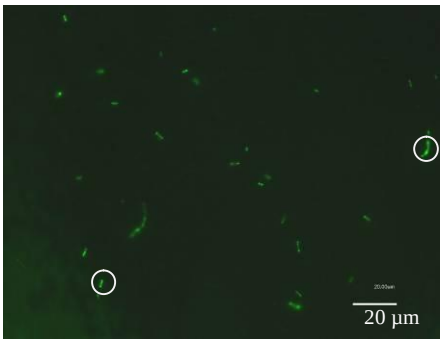
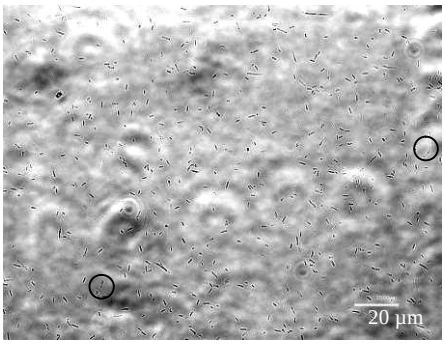
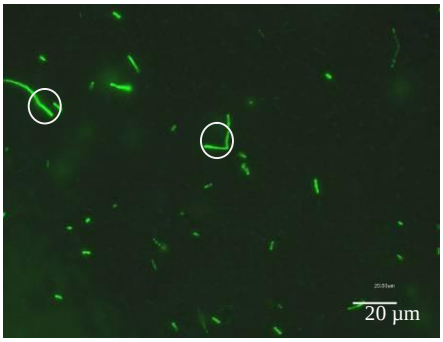
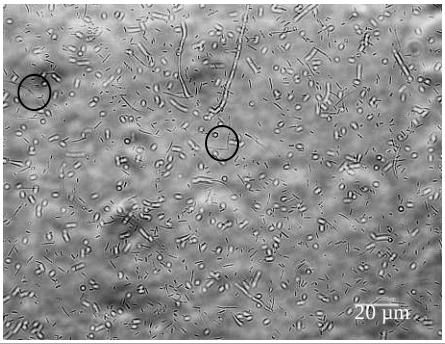
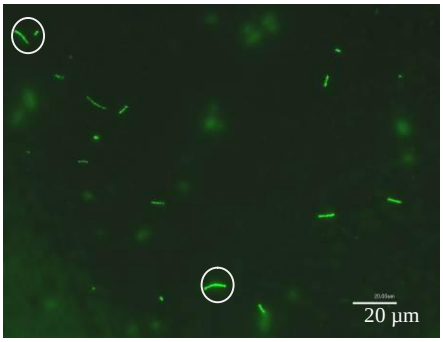
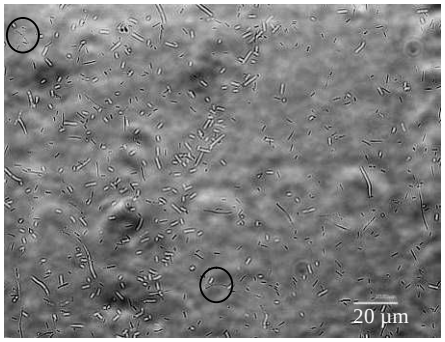
E. coli K12 Aptamers 50 pmol	Fluorescence, green light (495 nm)	Visible light
1AptK12		
2AptK12		
4AptK12		
6AptK12		

Figure 5.9 FAM-labelled aptamers 1AptK12, 2AptK12, 4AptK12 and 6AptK12 (50 pmol) binding to the surface of *E. coli* K12. Pictures were taken with a fluorescence microscope with 60× magnification with a green (495 nm) and visible light. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

5.3.4.2. Specificity of the aptamers

Aptamers are specific to their target (Brockstedt *et al.*, 2004; Hamula *et al.*, 2008; Joshi *et al*., 2009). The specificity of the aptamer pools was previously tested (4.3.4). The specificity of the individual aptamer sequences 1AptK12, 2AptK12, 4AptK12 and 6AptK12 was tested as a mixture and separately with the individual aptamers. A mixture of the aptamers (50 pmol) was incubated with overnight grown *E. coli* B and *S. aureus.* The positive control sample used was an overnight grown *E. coli* K12 culture. The mixture of the aptamers was used instead of individual aptamers. *L. acidophilus* was previously used to test the binding of the aptamer pool 9 (4.3.4.4), but the culture was not growing in the broth and was therefore left out from this study. After the incubation, samples were washed and the fluorescence was measured by the plate reader. The fluorescence values are presented in Figure 5.10. The results showed that the fluorescence measured for *E. coli* K12 was significantly higher than *E. coli* B and *S. aureus* (F=626.1, p=1.08×10^{-7}), even though some fluorescence was detected for *E. coli* B and *S. aureus.* By comparing these results to the specificity tests performed for aptamer pool 9 (4.3.4.1) it can be seen that the original aptamer amounts were lower. In that experiment, some fluorescence was also detected in *E. coli* B and *S. aureus* when 30 pmol aptamers were added. In this experiment, 50 pmol aptamers were used and higher fluorescence values were detected. This result confirms the aptamers are specific to *E. coli* K12 but a little binding to *E. coli* B and *S. aureus* can be detected when the aptamers concentration is high.

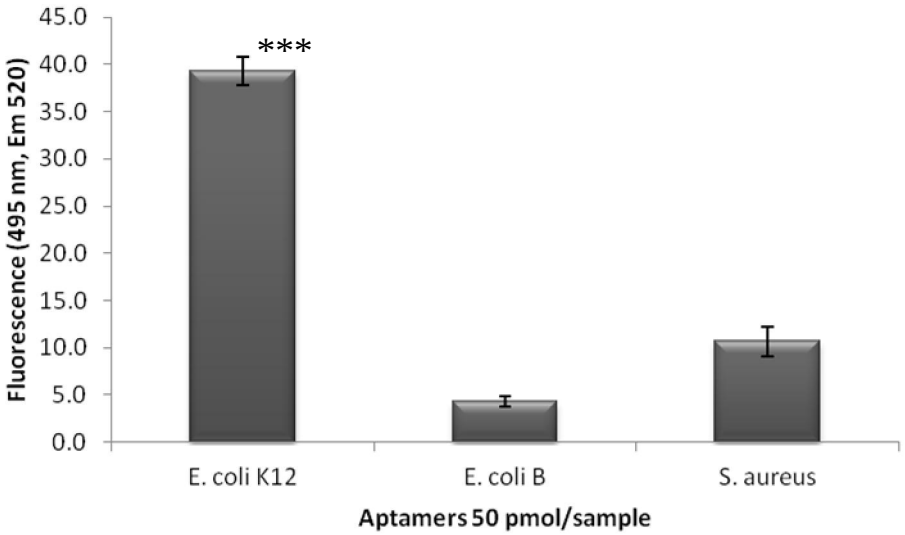


Figure 5.10 A mixture of FAM-labelled aptamers (1AptK12, 2AptK12, 4AptK12 and 6AptK12) binding to *E. coli* K12, *E. coli* B and *S. aureus*. The fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (*E. coli* K12=19, *E. coli* B=5, *S. aureus*=3). (F=626.1, p=1.08×10^{-7}). ***= Very highly significant (P<0,001).

The samples were visualised under the microscope with a fluorescent and visible light. The microscope images are presented in Figure 5.11. The fluorescence images for background samples (no aptamers added, 0 pmol) are on the left hand side, the images taken with fluorescent light in the middle and the visible light images on the right hand side. Bright green dots with a dark background can be detected in the positive control sample with *E. coli* K12. Not as bright and not as many dots can be seen in samples with *E. coli* B and *S. aureus*. This result confirms the aptamers 1AptK12, 2AptK12, 4AptK12 and 6AptK12 are specifically binding to *E. coli* K12 but a little binding to *E. coli* B and *S. aureus* can be seen. It can be seen on the fluorescence pictures that the background is darker when more fluorescence is present in the sample (*E. coli* K12) even though the same exposure time was used in all pictures (200 ms). It can be possible that the microscope is changing the lightning when the fluorescence of the samples is less bright.

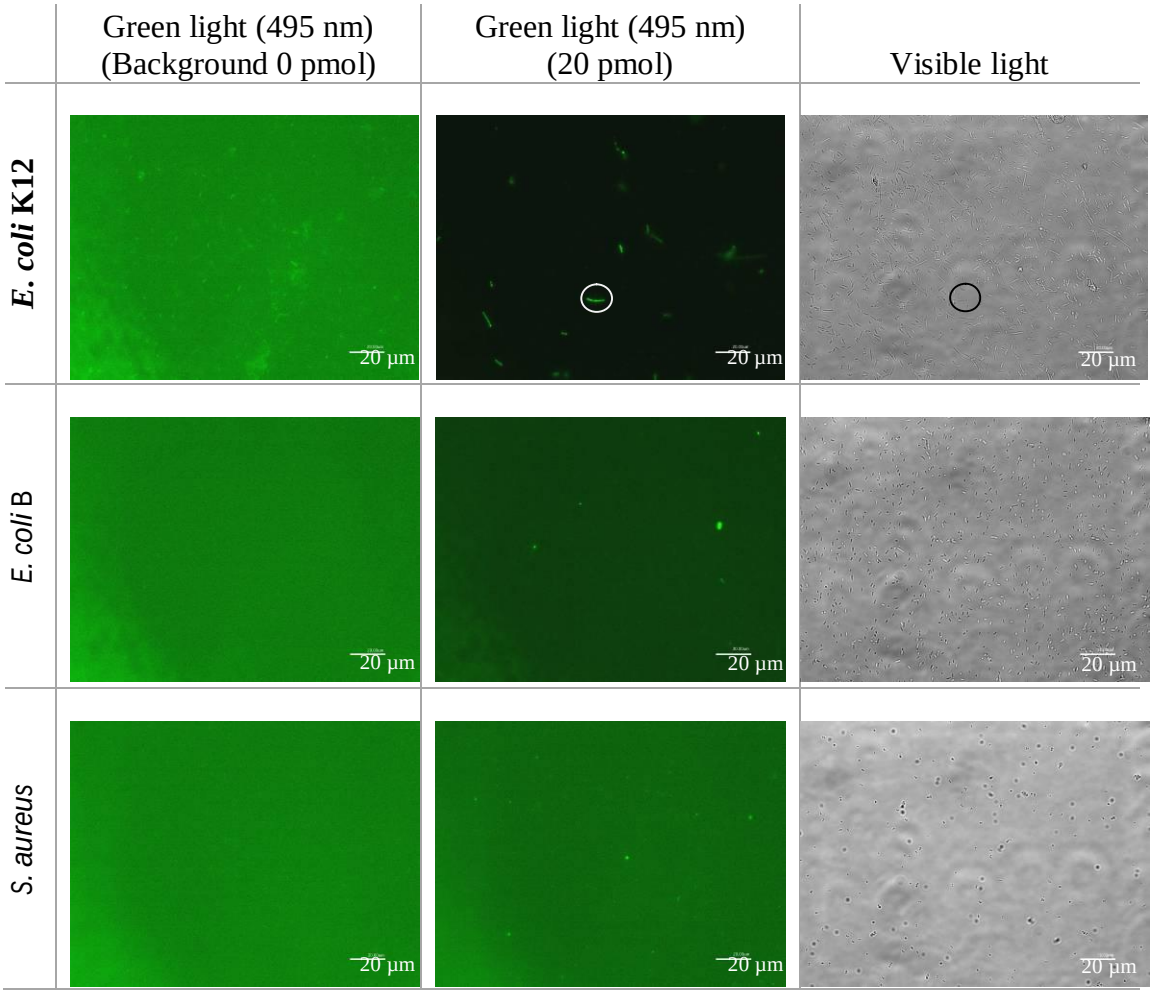


Figure 5.11 Aptamer specificity. A mixture of FAM-labelled aptamers (50 pmol) 1AptK12, 2AptK12, 4AptK12 and 6AptK12 incubated with the positive control *E. coli* K12, and the test strains *E. coli* B and *S. aureus*. Negative control samples (0 pmol) were performed with no aptamers. Pictures were taken with a fluorescence microscope with 60× magnification with a green light (495 nm, Em 520) and visible light. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

The specificity of the individual four aptamers 1AptK12, 2AptK12, 4AptK12 and 6AptK12 was tested against *E. coli* B and *S. aureus*. *E. coli* K12 was used as a positive control. The aptamer concentration used in this experiment was 20 pmol because this amount is shown to be enough to see the differences in the fluorescence values. Due to a technical problem with the plate reader no fluorescence readings could be measured with the sensitivity of 50 and therefore sensitivity 75 was used. These fluorescence values cannot be compared to the values of the previous experiments. The fluorescence values measured with a sensitivity of 75 are presented

in Figure 5.12. The general overview of the results is that the fluorescence is higher for *E. coli* K12 samples than for *E. coli* B or *S. aureus.* The *E. coli* K12 values for all samples 1AptK12 (F=23.9, p=1.3×10^{-3}), 2AptK12 (F=4.9, p=0.05), 4AptK12 (F=5.06, p=0.05) and 6AptK12 (F=5.49, p=0.04) are significantly higher than for *E. coli* B or *S. aureus.* The highest fluorescence was measured for 1AptK12 even though the binding analysis (5.3.4.1) showed the aptamer 6AptK12 has the highest and the aptamer 4AptK12 the second highest fluorescence when 20 pmol aptamers were added as seen in Figure 5.8. There can be several reasons for the different results between the different measurements. The number or bacterial cells might vary as the bacterial culture is overnight grown and therefore the age of the cultures vary. Also, as observed before, the washing steps might effect to the fluorescence readings. Sometimes more bacterial cells are washed off during the washes. The samples were looked under the microscope. Some bright dots were visible on the microscope images as seen in the images presented in Figure 5.11. The fluorescence images for these samples are not shown here as the results are similar to the results when the mixture of the aptamers was incubated with the bacterial cells in Figure 5.11.

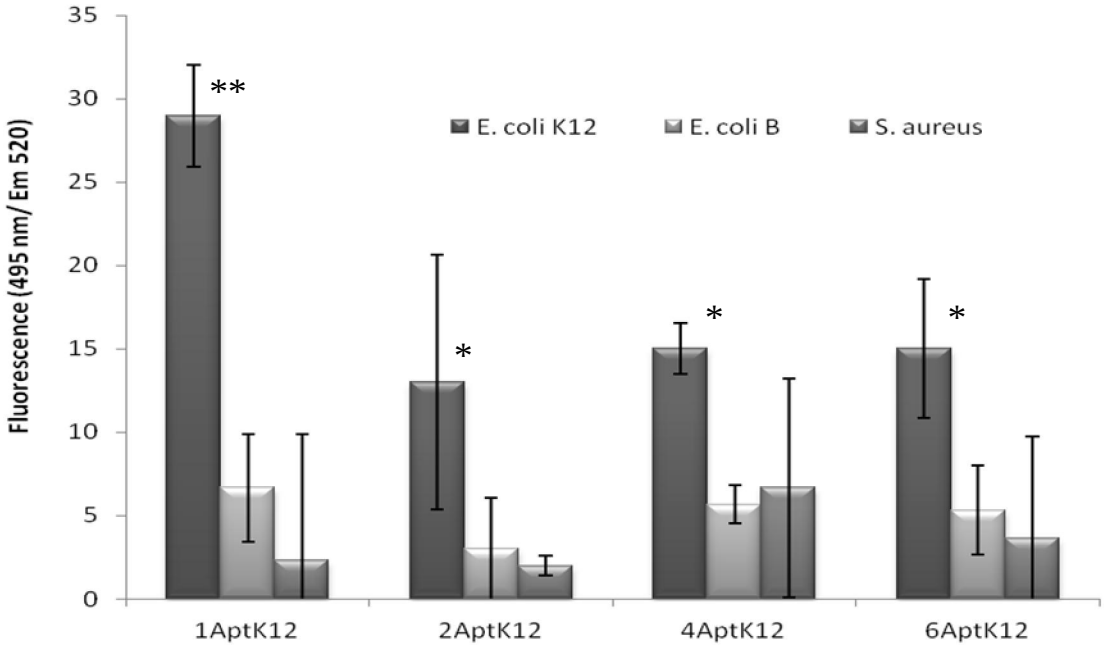


Figure 5.12 FAM-labelled aptamers 1AptK12, 2AptK12, 4AptK12 and 6AptK12 (20 pmol) binding to *E. coli* K12, *E. coli* B and *S. aureus.* The fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (*E. coli* K12=28.3; *E. coli* B=10.7; *S. aureus*=10.3). **Highly significant (P<0,01), *Significant (P≤0,05).

5.3.4.3. Aptamer binding to *E. coli* K12 at different ages

The molecules expressed on the surfaces of the bacterial cells may change with age, which may in turn influence the binding efficiency of the aptamers. Furthermore, it is not known which structures on the *E. coli* K12 cells the aptamers are binding. It was therefore considered useful to evaluate whether of the aptamers produced during this study exhibited changes in their binding as the age of the bacterial cultures changed and the number of the bacterial cells increased. To examine this further, *E. coli* K12 cultures were grown for 4, 8, 16, 20, and 24 hours and the aptamers (20 pmol) added to the bacterial cells.

The samples were visualised under the microscope with both green fluorescence and visible light. The microscope images are shown in Figure 5.13. The fluorescence images are on left hand side and the visible light images on right hand side. In the 4h sample, only one fluorescence dot can be seen and in the 8h sample, only two dots are visible. In the samples 16h, 20h and 24h more bacterial cells are detected. The 16h and 20h samples have similar amount of green dots and in sample 24h the number of dots is higher. When comparing the fluorescence pictures (left hand side) to the pictures taken with a visible light (right hand side), it can be seen that the number of bacterial cells is low in the 4h sample and only some fluorescence dots can be detected. The number of bacteria is at least twice as high as in the 8h sample and more fluorescence dots can be seen. In the 16h and 24h samples the bacterial cell number has increased noticeably and so has the number of the fluorescence dots. The background of the fluorescence images 4h and 8h is brighter than in samples 16h, 20h and 24h. This was also previously demonstrated as shown in Figure 5.11 where the images are brighter when none or a few green dots are visible. It remains unclear why the aptamers bind to only some of the bacterial cells but not to all of them. More detailed studies should be done to find out which molecules the aptamers are binding on the surface of bacteria. The bacterial cell number has to be equal in each sample in order to properly compare the samples. This study demonstrated that the more bacterial cells in the sample more green dots can be seen in the microscopy image.

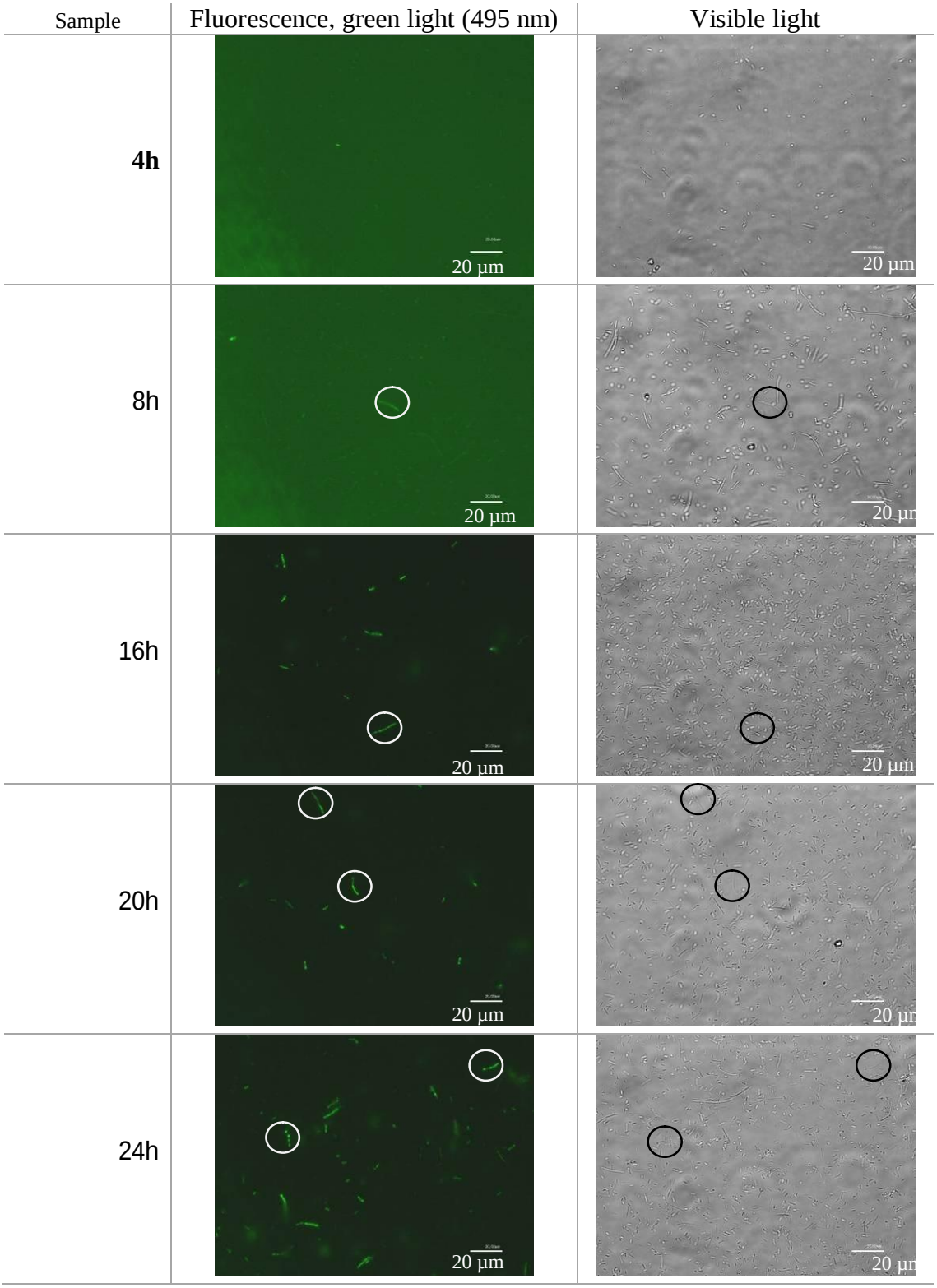


Figure 5.13 Aptamer binding to *E. coli* K12 at different ages. A mixture of FAM-labelled aptamers (20 pmol) 1AptK12, 2AptK12, 4AptK12 and 6AptK12 incubated with *E. coli* K12 culture 4h, 8h, 16h, 20h and 24h. Pictures were taken with a fluorescence microscope with 60× magnification with a green light (495 nm, Em 520) and visible light. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

5.4. CONCLUSIONS

The aptamer pool 9 specific to *E. coli* K12 was successfully selected and the binding of the aptamer was showed to the target. Since the aptamer nucleotide sequences of the aptamer pools were undefined the individual aptamers could not be synthesised. In this study aptamer pool 9, which binds specifically to *E. coli* K12, was cloned to plasmid vector using a commercial cloning kit. The cloned inserts were transformed into competent cells where the aptamer inserts were enriched. After a successful cloning the plasmid vectors were extracted and sequenced. The sequenced aptamers were analysed using predicted aptamer secondary structures and the ΔG values. Four aptamer sequences were synthesised with a fluorescence label and they showed specific binding to *E. coli* K12 cells.

In this study, four aptamer sequences were successfully identified and synthesised and the binding of these sequences to the target was shown. More aptamers could have been sequenced and identified in order to get a larger number of aptamer sequences. This could have led to even higher affinity aptamers. The preliminary study of aptamer binding to bacterial cells in different ages did not give the information that was needed to find out if the age of the bacterial cells effects to the aptamer binding. The study should be repeated with the equal bacterial cells numbers. This could lead to a number of experiments that can be carried out to evaluate the aptamer target molecules on the surface of *E. coli* K12 bacterial cells.

Evaluation of the performance of the aptamer pool 9 and the individual aptamers that are shown their binding to *E. coli* K12 in real food sample will be attempted. Natural yogurt containing probiotic micro-organisms will be used as a complex sample matrix in these experiments. The aptamer selection methods developed will be used to select the aptamers to bind to pathogenic micro-organisms.

CHAPTER 6 – Aptamer detection of *Escherichia coli* K12 from natural yogurt

6.1. INTRODUCTION

Aptamers were selected to bind live *E. coli* K12 cells. The binding properties of aptamer pool 9 (4.3) were analysed and the aptamer sequences were subsequently cloned (5.3.1). The binding of the identified aptamers to *E. coli* K12 and their specificity were tested (5.3.4). The aptamers were shown to be specifically binding to *E. coli* K12.

Aptamers selected in this study have shown to be working in buffer conditions. It has been demonstrated that the aptamers can detect microbes in more complex matrices such as in food (Bruno, 2009; Bruno *et al.*, 2009; Joshi *et al.*, 2009). These studies represent the only reports available where the aptamers have been used in food matrices. In this study, aptamers were tested to see if they can be used to detect bacterial cells from a real food system. The activity of the specific *E. coli* K12 aptamer pool 9 was first tested in tap water and the results were used to develop a detection assay that can be used for the detection of bacteria in yogurt. Fluorescent labelled aptamer pool 9 which were known to bind to *E. coli* K12 was then used to detect the bacterial cells in natural yogurt that contained *Lactobacillus acidophilus*, and *Bifidobaterium* spp., and for the test sample were also spiked with E *coli* K12. Once the aptamer pool 9 was cloned the detection of *E. coli* K12 from probiotic yogurt was also demonstrated with the individual aptamers.

6.2. METHODS

6.2.1. Aptamer activity in water

Aptamers (pool 9) selected to bind *E. coli* K12 have been used to detect live bacterial cells in buffer conditions. To see if the aptamers retained their activity in unbuffered conditions and can still be used for bacterial detection tap water was used as the matrix. Tap water was spiked with an excess of *E. coli* K12 bacterial cells. The overnight grown bacterial suspension (1 ml) was centrifuged (2.3.9.1) and the bacterial pellet was resuspended into 1 ml of tap water. The aptamers (10 pmol and 20 pmol) were added into the solution and incubated for 45 min. The control sample

without the aptamers was also prepared. Samples were washed twice with BB and the fluorescence was measured by a plate reader.

6.2.2. Detection of *E. coli* K12 from probiotic yogurt

6.2.2.1. Method development

A method was developed to investigate whether the aptamers from pool 9 could be used to detect bacterial cells in a more complex food matrix. Natural probiotic yogurt that contained live cultures of *L. acidophilus* and *Bifidobacterium* spp. was used as the food matrix. Fluorescent FAM-labelled aptamers were produced (2.3.7) and an overnight culture of *E. coli* K12 (10 ml) was prepared (2.3.9.1) and resuspended into 4 ml of binding buffer (BB). Yogurt samples were prepared by mixing 3 ml of natural probiotic yogurt and 3 ml of BB for negative samples and 3 ml of yogurt, 2 ml of BB and 1 ml of bacterial suspension for the samples. Samples were mixed followed by centrifugation at 1500*g* for 10 minutes to separate the bacteria from the yogurt. The supernatant, containing the bacterial cells, was collected and centrifuged at 3500*g* for 5 minutes. The bacterial pellet was washed twice with BB and then resuspended into 100 µl of BB containing 20 pmol of fluorescence labelled aptamers. The aptamers were bound to the bacterial cell surface (2.3.9.1) and the fluorescence of the samples was read by the plate reader (2.3.9.3). The fluorescence values were compared by one way analysis of variance (ANOVA).

6.2.2.2. Detection

Further optimisation for the detection method was necessary in order to detect the *E. coli* K12 cells from yogurt with the aptamers. The sample sizes were reduced and less *E. coli* K12 bacterial cells added making it possible to perform this experiment in microcentrifuge tubes. The FAM-labelled aptamers were produced (2.3.7) and the bacterial cells were prepared (2.3.9.1). The bacterial suspension (3 ml) was washed and re-suspended into 1500 µl of BB. The yogurt samples were 750 µl of probiotic yogurt and 750 µl of BB for the negative control samples. The *E. coli* K12 spiked yogurt samples were 750 µl of yogurt and 500 µl of bacterial suspension topped up with BB to the final volume of 1000 µl. The samples were mixed well and the bacteria were separated from the yogurt by centrifuging the samples for 5 minutes at 1000*g*. This centrifugation speed and time was lowered from the time and speed previously used (1500*g*, 10 min) such that more bacterial cells could be separated.

The supernatant, which contained bacterial cells, was collected. To recover more bacterial cells from the yogurt mixture, an additional 200 µl of BB was added and after samples were mixed they were centrifuged as above. The supernatant was collected and added to the samples. The samples were now centrifuged at 3500*g* for 5 minutes and washed twice with BB. The bacterial cells were resuspended into 100 µl of BB with 20 pmol FAM-labelled aptamers. Control samples with no aptamers were done by resuspending the cells into 100 µl BB. The samples prepared for analysis are summarised in Table 6.1. The fluorescence values were read by the plate reader (2.3.9.3) and the results were compared by one way ANOVA.

Table 6.1 Yogurt samples.

Sample	*E. coli* K12	Aptamers (20 pmol)
0.0	-	-
0.1	+	-
1.0	-	+
1.1	+	+

6.2.3. Detection of *E. coli* K12 from yogurt with FAM-labelled aptamers

The cloned four individual aptamers specific to *E. coli* K12 (5.3.3) were synthesised with FAM-labels. The yogurt samples were prepared as described above (6.2.2.2) and the bacterial cells were extracted from the yogurt. A mixture of the aptamers contained 5 pmol of each of the four cloned FAM-labelled aptamers (20 pmol in total) was incubated with the samples in triplicate. Samples were incubated for 45 min and after the washes the fluorescence was measured by the plate reader. The fluorescence values were compared by one way ANOVA.

6.3. RESULTS AND DISCUSSION

6.3.1. Aptamer activity in water

The aptamers have been shown to be working in optimal buffer conditions. The activity of the FAM-labelled aptamer pool 9 was first tested in tap water which had been spiked with *E. coli* K12 bacterial cells to test if the aptamers activity remains unchanged in these conditions. The fluorescence intensity values are presented in

Figure 6.1. Two aptamer concentrations were used (10 pmol and 20 pmol) and it can be seen in the graph that the fluorescence detected for 10 pmol sample is almost half of the fluorescence detected for the 20 pmol sample where the aptamer amount was twice as much. This result shows that the aptamers were active in water and that they can be used to detect bacterial cells in water samples.

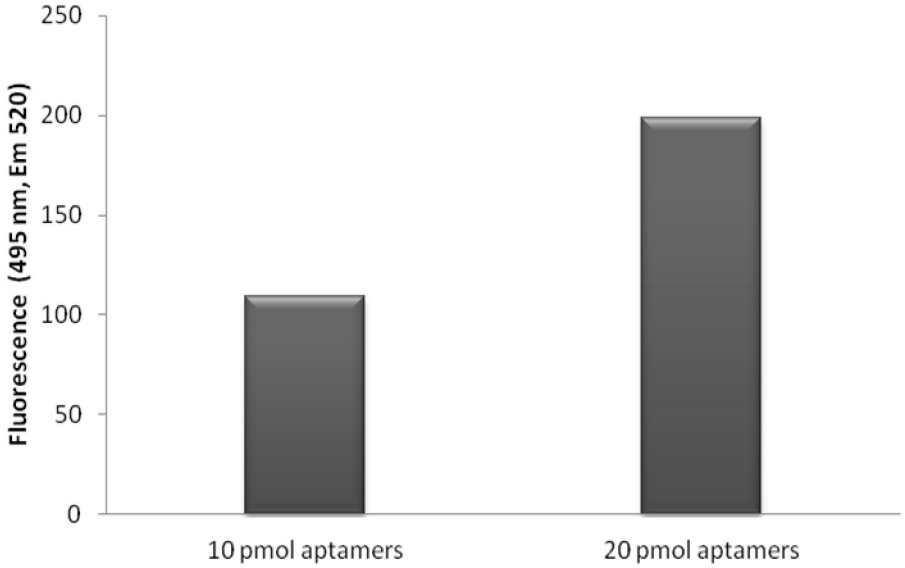


Figure 6.1 FAM-labelled aptamer pool 9 binding to *E. coli* K12 in tap water. The fluorescence (495 nm, Em 520) was measured by a plate reader (n=1). The values are corrected for background (67.0).

6.3.2. Detection of *E. coli* K12 from probiotic yogurt

6.3.2.1. Method development

The method to detect the aptamers from natural yogurt was tested. Yogurt was spiked with *E. coli* K12 and the bacterial cells were extracted from yogurt followed by the fluorescence aptamer detection of *E. coli* K12. The fluorescence values are presented in Figure 6.2. It can be seen that the sample where bacterial cells have been added has significantly higher fluorescence than the sample where no *E. coli* K12 cells have been added (F=34.27 and P=4.2×10^{-3}). The background fluorescence was not taken in to account because the control samples without the aptamers were not included in this experiment. This result, however, demonstrates that the aptamers were active and could be used to detect live bacterial cells present in yogurt samples. The results also showed that the aptamers were not binding to the other bacteria

present in yogurt. It has previously been demonstrated that these aptamers do not bind to *L. acidophilus* (4.3.4.3).

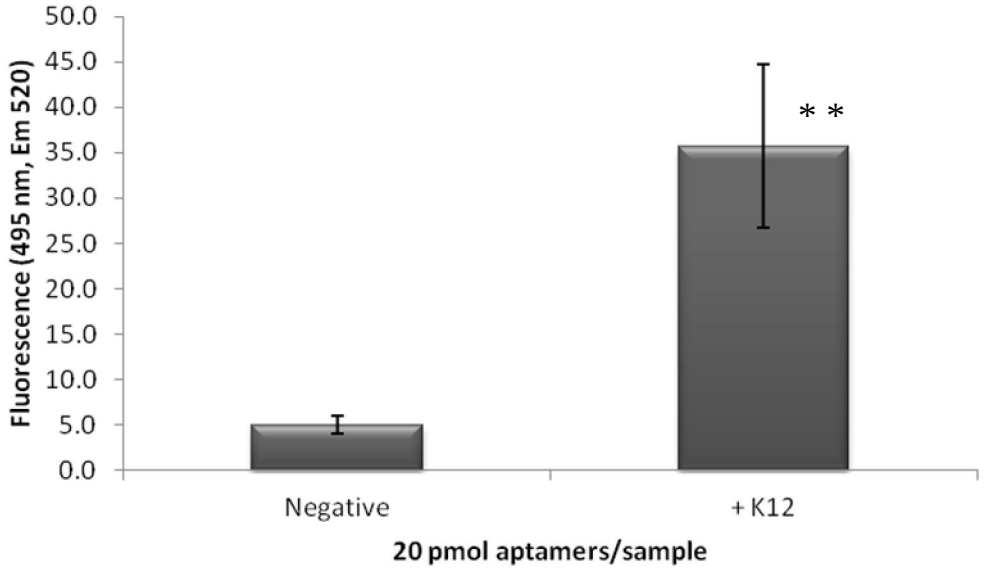


Figure 6.2 FAM-labelled aptamer pool 9 binding to *E. coli* K12 extracted from yogurt. The fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. The samples are corrected for background (124.0). **Highly signicicant (p<0.01).

6.3.2.2. Detection of the *E. coli* K12 from yogurt with specific aptamer pool 9

The aptamer detection of *E. coli* K12 from yogurt was further optimised. The experiment was done with smaller sample sizes and more control samples so that the background fluorescence could be reduced. The fluorescence values are presented in Figure 6.3. It can be seen that the samples where no *E. coli* K12 has been added (Negative) have a significantly lower fluorescence than for the *E. coli* K12 sample (F=18.6, P=0.05). This result proves that the specific aptamer pool can be used to detect live bacterial cells from yogurt but the detection limit of the technique needs to be determined.

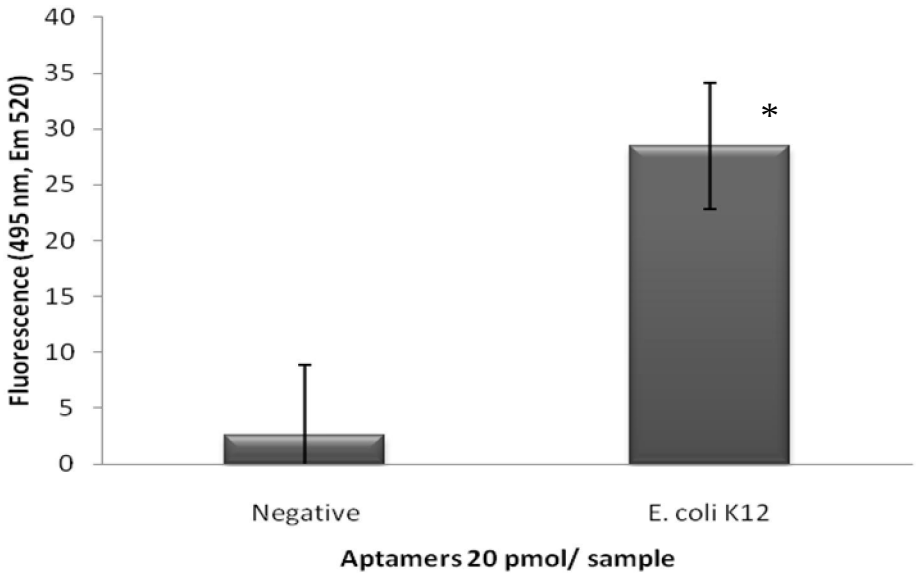


Figure 6.3 FAM-labelled aptamer pool 9 binding to *E. coli* K12 extracted from yogurt. The fluorescence (495 nm, Em 520) was measured by a plate reader (n=2). The values are presented as means ± s.d. The values are corrected for background (Negative=2.0; *E. coli* K12=8.5). *Significant ($P \leq 0.05$).

6.3.3. Detection of *E. coli* K12 from yogurt with FAM-labelled aptamers

Cloned aptamers were used to detect *E. coli* K12 from yogurt samples containing probiotic strains *L. acidophilus* and *Bifidobacterium* spp. The fluorescence was measured by the plate reader and the values are presented in Figure 6.4. It can be seen in the figure that the fluorescence of the sample, where *E. coli* K12 has been added, is significantly higher than the fluorescence in the negative sample (F=36.75, $P=3.7\times10^{-3}$). Relatively high fluorescence was measured for the negative control sample even though the fluorescence was significanly lower than the fluorescence of the *E. coli* K12 sample. The bacterial cells, including the bacteria in yogurt, were extracted and it might be possible that some components from yogurt have remained in the samples and the aptamers have bound to them. It is also possible that the unbound aptamers were not washed off properly. One possibility is that some binding to *Bifidobacterium* spp. takes place and therefore some fluorescence can be detected. Nonetheless, the results presented here show that the fluorescent-labelled aptamers can be used to detect live bacterial cells from yogurt.

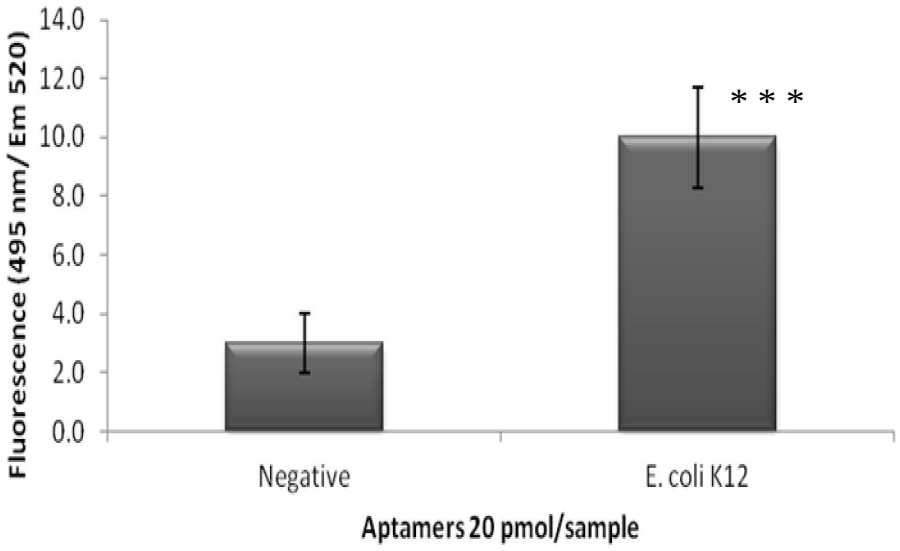


Figure 6.4 FAM-labelled aptamers 1AptK12, 2AptK12, 4AptK12 and 6AptK12 binding to *E. coli* K12 extracted from natural probiotic yogurt. The fluorescence (495 nm, Em 520) was measured by a plate reader (n=3). The values corrected for background (Negative=7.0; *E. coli* K12=8.0).The values are presented as means ± s.d. (F=36.75, P= 3.7×10^{-3}). *** Very highly significant (P<0,001).

6.4. CONCLUSIONS

Detection of live *E. coli* K12 bacterial cells with specific aptamers has been previously demonstrated in buffer conditions (4.3.1; 5.3.4.1). In this study, the binding of these specific aptamers was tested in tap water and in natural yogurt samples. First, the activity of the aptamer pool in water was tested and the results demonstrated that the aptamers were active in water. The activity of the aptamer pool and the individual aptamers was also tested in bacterial cells extracted from yogurt. *E. coli* K12 bacteria cells extracted from yogurt were successfully detected by the aptamers. The detection limit of the technique would give us more information about the sensitivity of the aptamers. There have been no reports of the use of aptamers to detect bacterial cells in yogurt in the scientific literature.

Aptamers were used to detect bacterial cells from water and yogurt making them very promising in a development of new type of detection method for food poisoning bacteria. In this study non-pathogenic *E. coli* K12 was used as an example strain but aptamers can also be used in a detection of pathogens. There is a strong possibility that the aptamers can be used to detect the bacterial cells in different types of food matrices, for example in solid matrices such as meat or cheese. Fluorescent-labelled

aptamers could possibly be added on the surface of the meat and the non-binding aptamers could be washed off. The fluorescence can then be measured or visualised. It is also possible to envisage the development of a wide range of assay procedures and formats based on the use of specific aptamers in a fashion analogous to the use of antibodies.

CHAPTER 7 – Selection of the aptamers against pathogenic bacteria

7.1. INTRODUCTION

Aptamers have been shown to be a potential tool in development of rapid detection methods for food-borne pathogens (Bruno *et al*., 2009; Joshi *et al*., 2009; Ohk *et al.,* 2010; Yamamoto *et al.,* 2010; Kärkkäinen *et al.,* 2011a). The aptamer selection method was further developed which enabled the production of molecules which demonstrated specific binding to non-pathogenic *E. coli* K12 live bacterial cells (3.3.2). The aptamers were then cloned (5.2.1), sequenced (5.2.3) and the binding and the specificity of these selected sequences were tested by using a method based on fluorescence (5.2.4).

The aptamer selection method was applied to the food-borne pathogenic bacteria (Kärkkäinen *et al.,* 2011a). The aptamers were selected to bind to two different pathogenic *E. coli* strains including O157, three *Listeria* strains (*L. innocua* and two species of *L. monocytogenes*) and two *Salmonella* strains (*S. typhimurium* and *S. enteritidis*). The aptamers against pathogenic *E. coli* were selected from an existing pool of *E. coli* K12 binding aptamers while the normal selection process where the aptamers are selected from a random DNA library (2.3.6) was used to select the aptamers against *Listeria* and *Salmonella*. From these aptamers two pools having the best characteristics were selected for further analysis. As a result of this study, three aptamer sequences for *E. coli* O157 and three sequences for *S. typhimurium* were identified and the binding was tested. Some of the sequences showed specific binding and good affinity against their target bacteria.

7.2. METHODS

7.2.1. Aptamer selection

A DNA library was produced as described before (2.2.3) and the aptamers were selected to bind to pathogenic bacterial strains. The selection of the aptamers was done following the protocol (2.3.6) with one exception; the counter selection was not performed for all strains. The counter selection was omitted in order to see if it was

necessary in terms of the specificity of the aptamers but also for time saving reasons to reduce the time used for the selection process.

7.2.1.1. Aptamers against *Escherichia* coli 496 and O157 497

Aptamers were selected to bind to two strains of pathogenic *E. coli* from pool 9 of *E. coli* K12 binding aptamers. It is possible the aptamer pool 9 binds to some structures on the surface of *E. coli* K12 that are the same as on the surface of the pathogenic *E. coli* strains. Selection was done as a normal aptamer selection (2.3.6) except that the selection process was only performed once. The selection and the counter selection were done as previously described (3.3.2). After the incubation the samples were washed three times. The aptamers were collected and amplified by PCR (2.3.1) and the samples were separated on agarose gel (2.3.2).

7.2.1.2. Aptamers against *Listeria innocua* and *Listeria monocytogenes*

The aptamers were selected from the random DNA library to bind to *L. innocua* 17, *L. monocytogenes* 489 and *L. monocytogenes* 490 as previously described (2.3.6). The aptamers were selected for all three strains until aptamer pool 7. The only successfully selected aptamer pool 7 was for *L. monocytogenes* 490. This sample was divided in two tubes and the aptamers were further selected in duplicate. To increase the PCR yield the template was added to the reaction in volume 1.5 µl instead of 1 µl. The aptamers were collected and amplified by PCR (2.3.1) and the samples were separated on agarose gel (2.3.2).

7.2.1.3. Aptamers against *Salmonella typhimurium* and *Salmonella enteritidis*

The aptamers were selected from a random DNA library to bind to *S. typhimurium* 223 and *S. enteritidis* 1152 following the selection protocol (2.3.6). The selection was repeated nine times. After each selection round the aptamers were collected and amplified by PCR (2.3.1) and the samples were separated on agarose gel (2.3.2).

7.2.2. Fluorimetry detection of the pathogen binding aptamer pools

The aptamer pools were fluorescent (FAM) labelled (2.3.7) and the binding of the aptamers was tested by fluorimetry (2.3.9.3). Four PCR products were mixed together, purified and used in the binding reaction. The binding was tested for the aptamers that were selected against *E. coli* 496, *E. coli* 497, *L. monocytogenes* 490,

S. typhimurium 223 and *S. enteritidis* 1152. The binding of the previously selected *E. coli* K12 aptamers (3.3.2) was tested in parallel. The DNA concentration measurement was not used in this study but the fluorescence of the aptamers was measured before they were mixed with the samples.

7.2.3. Cloning of the aptamers

To find out the specific sequences of the aptamers, the ninth aptamer pools against *E. coli* O157 497 and *S. typhimurium* 223 were selected for cloning. The pGEM-T Easy vector cloning was done as previously described (2.3.12.1) and the same protocol was followed as for the cloning of *E. coli* K12 aptamers (5.2.1).

7.2.3.1. Ligation and transformation

Aptamer pool 9 against *E. coli* O157 497 and *S. typhimurium* 223 were ligated into the plasmid vector (2.3.12.1). In this study, three different insert : vector ratios were used (5.2.1.1) for the ligation as in the ligation for the *E. coli* K12 aptamers. The components for the reaction are presented in Table 5.1. From the indicator plates, five positive white and one negative blue colony were randomly selected and streaked on new indicator plates. This was done for both, *E. coli* and *S. typhimurium* samples. The plates were incubated overnight.

7.2.3.2. PCR analysis for positive colonies

The overnight grown colonies were analysed. It has previously been demonstrated that the colour selection is not reliable method for selecting the positive colonies. The PCR analysis was demonstrated to be a fast, easy and reliable way of analysing the positive colonies (5.3.1.2). The PCR analysis of the samples was performed for all of the colonies on the indicator plates (2.3.12.2; 5.3.1.2). Among the PCR control a negative control, that contained the PCR primers and a colony with a control insert (positive control for cloning), was done.

7.2.4. Sequencing of cloned vectors and aptamer analysis

The aptamer sequences were determined by sequencing the plasmid vectors with inserted aptamers. The colonies that were shown to be positive in PCR analysis were inoculated into LB-media and the plasmid was purified from the overnight grown

culture (2.3.12.4). Six plasmid DNA samples (30 µl), three of each strain, were sequenced (2.3.12.6). The 100 nt long aptamer sequences were identified from the vector sequence.

Aptamer secondary structures make the binding of the aptamer to their target possible. The structures were analysed as previously described (2.3.12.7). Three aptamers for *E. coli* O157 497 and three aptamers for *S. typhimurium* 223 were selected and synthesised with a fluorescent FAM label. These aptamers were selected because of their matching structure to the original 100 nt secondary structure. The energy needed to break down the structure (ΔG) that also represents the strength of the structure was taken into account when selecting the aptamers to be synthesised.

7.2.5. Binding of the cloned aptamers

Three cloned and synthesised FAM-labelled aptamers for both strains *E. coli* O157 497 (1Apt497, 2Apt497 and 4Apt497) and *S. typhimurium* 223 (2Apt223, 3Apt223 and 5Apt223) were tested. The aptamers (10 pmol, 20 pmol and 50 pmol) were incubated with bacterial suspension in triplicate and the fluorescence was analysed by fluorimetry and by visualising the samples under the fluorescence microscope (2.3.9). The fluorescence values were compared by one way analysis of variance (ANOVA).

7.2.5.1. *E. coli* O157 aptamers

The binding of the fluorescent-labelled aptamers 1Apt497, 2Apt497 and 4Apt497 against *E. coli* O157 497 was tested as described above (7.2.5).

7.2.5.2. Binding of *E. coli* O157 aptamers to *E. coli* K12

The aptamers to bind to *E. coli* O157 497 were selected from a pool of *E. coli* K12 binding aptamers. The binding of these aptamers against *E. coli* K12 was tested by incubating 20 pmol of the aptamer 1Apt497, 2Apt497 and 5AptK12 with *E. coli* K12 bacterial cells and then measuring the binding by fluorimetry test (2.3.9.3). *E. coli* K12 specific aptamer 4Apt497 was used as a positive control. The fluorescence was measured with a sensitivity of 75 instead of 50. The fluorescence values were analysed using the statistic test ANOVA. The samples were then visualised and the

images were taken under the fluorescence microscope with a 60× magnification (2.3.9.2).

7.2.5.3. *S. typhimurium* binding aptamers

The binding of the fluorescent-labelled aptamers 2Apt223, 3Apt223 and 5Apt223 against *S. typhimurium* 223 was tested as described above (7.2.5).

7.2.5.4. Specificity of the *S. typhimurium* binding aptamers

The specificity of the *S. typhimurium* binding aptamers 2Apt223, 3Apt223 and 5Apt223 was tested. The aptamers (20 pmol) were incubated with *E. coli* K12, *L. plantarum, S. enteritidis* and *S. typhimurium* 223 (2.3.9.1) in triplicate. After the incubation, the samples were washed and the fluorescence was measured by the plate reader (2.3.9.3) and the samples were visualised under the fluorescence microscope (2.3.9.2). The fluorescence values were compared by one way ANOVA.

7.3. RESULTS AND DISCUSSION

7.3.1. Aptamer selection

The aptamers were selected from the random DNA library that has previously been created (3.3.1) against some common food poisoning bacteria (European Food Safety Authority, 2011). An agarose gel image of the DNA library can be seen in Figure 3.3.

7.3.1.1. Aptamers against *Escherichia* coli 496 and O157 497

Aptamer pool 9 was previously selected to bind to *E. coli* K12 (3.3.2) and the binding of this pool to its target has previously been shown (3.3.4; 4.3.1). In this study, the aptamers were selected to bind to pathogenic *E. coli* 496 and *E. coli* O157 497 from the *E. coli* K12 binding aptamer pool 9. The agarose gel image is presented in Figure 7.1. It can be seen that all the aptamer pools (1-2: *E. coli* 496 and 3-4: *E. coli* O157 497) have strong bands. The amplification of these samples indicates that the aptamers have bound to the pathogenic strains of *E. coli*. The bacterial control samples are in lanes 5 and 6. No amplification can be detected in these samples as expected. A faint band can be seen in the DNA control sample in lane 7. This

indicates some aptamers that are not necessarily binding to *E. coli* are remaining in the tube after the washes.

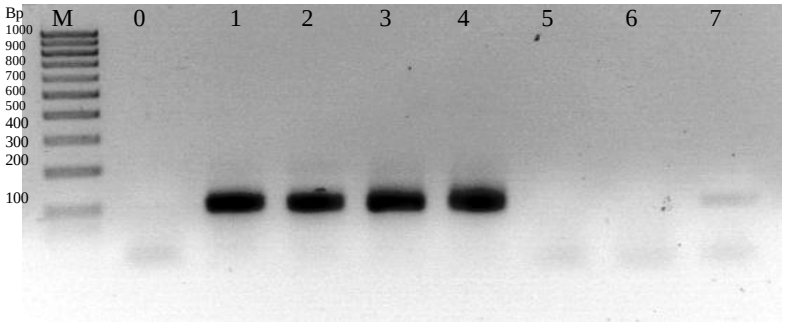


Figure 7.1 2 % Agarose gel with aptamer pool for *E. coli* 496 (lanes 1 and 2) and *E. coli* O157 497 (lanes 3-4). The bacterial control samples are in lanes 5 and 6 and the DNA control is in lane 7. The lane M on the gel is a PCR Sizer 100bp DNA Ladder and the 0 is the PCR control.

7.3.1.2. Aptamers against *Listeria innocua* and *Listeria monocytogenes*

Aptamers were selected to bind to three different *Listeria* strains: *L. innocua* 17, *L. monocytogenes* 489 and *L. monocytogenes* 490. Only seven rounds of selection were repeated for *L. innocua* 17 and *L. monocytogenes* 489, because no PCR amplification could be seen after the sixth round of selection. For *L. monocytogenes* 490, nine rounds of selection were performed. After each round the PCR products were separated on agarose gel to see the 100 bp product. The selection of the aptamer pools 1, 2, 3 and 4 was successful even though the PCR resulted only in faint bands on agarose gels. The gel images are not presented here. In Figure 7.2 is an agarose gel images of the aptamer pools 5, 6 and 7. It can be seen that the aptamers for *L. innocua* 17 in lanes 1 and 2 on gel 7.2a, 7.2c and 7.2d (black boxes) have faint bands. The bands can also be seen in the *L. monocytogenes* 489 aptamers in lanes 1 and 2 on gel 7.2b and lanes 3 and 4 on gel 7.2c and 7.2d (grey boxes). Aptamers for *L. monocytogenes* 490 in lanes 3 and 4 on gel 7.2b (white boxes) have the strongest bands. For aptamer pool 6 and 7 only one of the 490 aptamers appears to have a strong band on the gel (lane 5 on gel 7.2c and 7.2d). This indicates there is more aptamer binding to the bacterial cells in this sample compared to the two other *Listeria* strains. This strong band was extracted, divided into two and used for further selection. The PCR control, where no aptamer template was added, is in lane 0 on

both of the gels 7.2a and 7.2b. The bacterial control and DNA control resulted in clear bands but the results are not shown in the gel images.

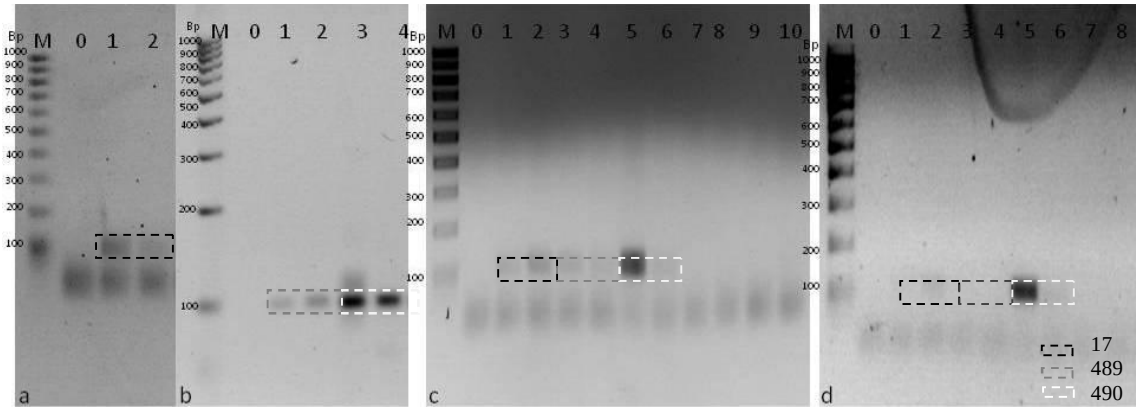


Figure 7.2 Aptamer pool 5 (a and b), 6 (c) and 7 (d) for *L. innocua* 17 (black boxes), *L. monocytogenes* 489 (grey boxes) and *L. monocytogenes* 490 (white boxes) on 2% agarose gel. The bacterial control samples are in lanes 7, 8 and 9 (c) and in lane 7 (d). The DNA control is in lane 10 (c) and 8 (d). The M on the gel is a PCR Sizer 100bp DNA Ladder and the 0 is the PCR control.

After the poor amplification of the aptamers against *L. innocua* 17 and *L. monocytogenes* 489 that can be seen in Figure 7.2 the further selection was only performed for *L. monocytogenes* 490. The agarose gels for aptamer pools 8 and 9 are shown in Figure 7.3. The amplification products are marked with white boxes in lanes 1 and 2. The bacterial and DNA control samples are on gel 7.3a in lanes 3 and 4. These control samples were also performed for the selection round 9 but the results are not shown here.

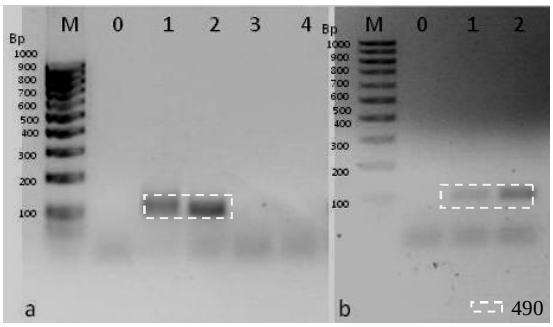


Figure 7.3 Aptamer pool 8 (a) and 9 (b) for *L. monocytogenes* 490 (white boxes) on 2% agarose gel. Lane M on the gel is a PCR Sizer 100bp DNA Ladder and lane 0 is the PCR control. Bacterial control is in lane 3 and the DNA control in lane 4 (a).

7.3.1.3. Aptamers against *Salmonella typhimurium* and *Salmonella enteritidis*

Aptamers were selected to bind to *S. typhimurium* 223 and *S. enteritidis* 1152. Nine rounds of selection were performed. The agarose gels of aptamer pool 1, 2, 3 and 4 are shown in Figure 7.4 and pool 5 in Figure 7.5. The aptamers for *S. typhimurium* 223 are in lanes 1 and 2 (black box) and the bacterial control sample is in lane 3. The aptamers for *S. enteritidis* 1152 are in lanes 4 and 5 (white box), and the bacterial control is in lane 6. The DNA control sample where no bacterial cells were added is in lane 7. It can be seen in Figure 7.4 that the bands for the *S. typhimurium* 223 aptamers are slightly more intense than the bands for the *S. enteritidis* 1152 aptamers. The higher PCR yield may indicate that from the original DNA library more aptamers have bound to *S. typhimurium* 223 than to *S. enteritidis* 1152. In Figure 7.5 the results for pool 5 are shown. The same trend is seen for this pool with *S. typhimurium* 223 bands being stronger than the bands for *S. enteritidis* 1152 except for the aptamer pool for *S. enteritidis* 1152 in lane 5 (Figure 7.5). This pool show a similar intensity as the *S. typhimurium* 223 pools but stronger than the pool in lane 4. The reason for this can be that more aptamers have bound to the bacterial cells and therefore the template concentration in PCR is higher. The PCR control samples (0) appear to be clear in all gels. No amplification can be seen in bacterial control samples (lanes 3 and 6). This result indicates the bacterial cells do not have the binding sites for the primers. The clear DNA control sample shows there were no free aptamers remaining in the solution or binding to the tube wall after the washes.

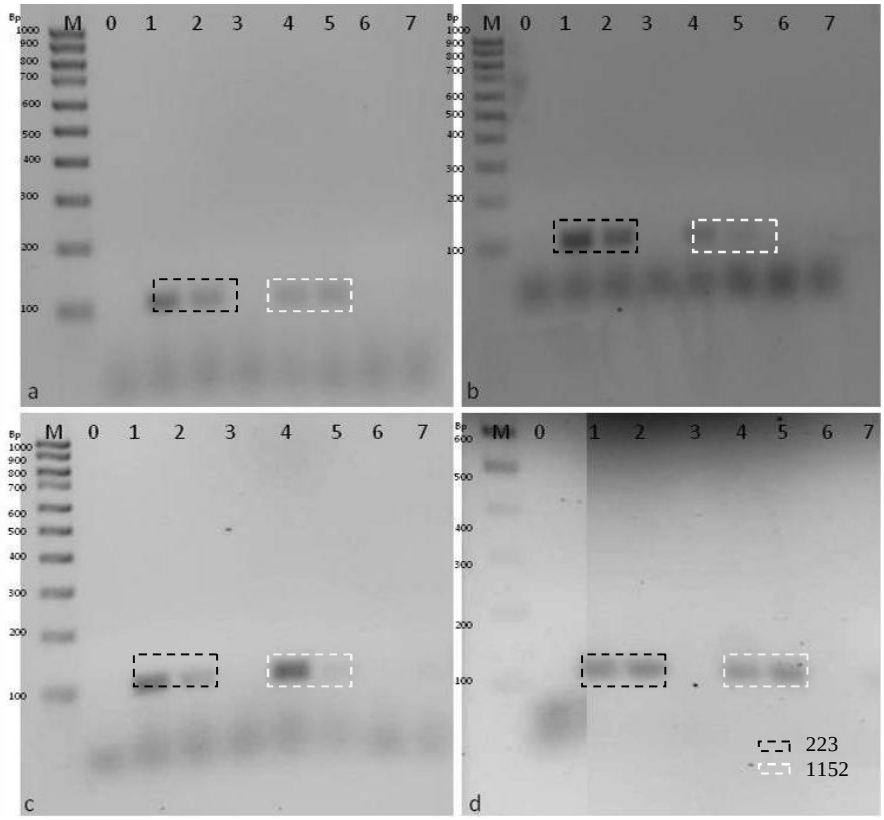


Figure 7.4 Aptamer pools 1 (a), 2 (b), 3 (c) and 4 (d) for *S. typhimurium* 223 (black boxes) and *S. enteritidis* 1152 (white boxes) on agarose gel (2%). Lane M is a PCR Sizer 100bp DNA Ladder and lane 0 is the PCR control. Bacterial control sample is in lane 3 for 223 and in lane 6 for 1152. The DNA control sample is in lane 7.

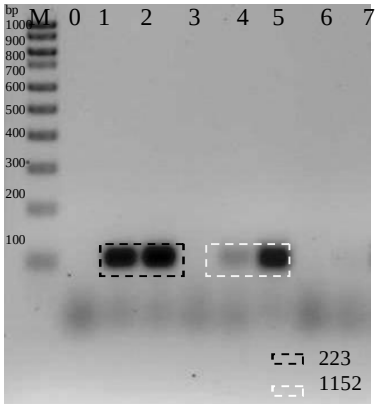


Figure 7.5 *S. typhimurium* 223 (black box) and *S. enteritidis* 1152 (white box) aptamer pool 5 on agarose gel (2%). The M on the gel is a PCR Sizer 100bp DNA Ladder and in lane 0 is the PCR control. Bacterial control samples are in lanes 3 and 6 and the DNA control sample is in lane 7.

The aptamer pools 6, 7, 8 and 9 are shown in Figure 7.6. The aptamers for *S. typhimurium* 223 are marked with black boxes and for *S. enteritidis* 1152 are in white boxes. The results show that the aptamer pools for *S. typhimurium* 223 (black boxes)

have stronger bands than the *S. enteritidis* 1152 for all the pools (white boxes). The PCR control samples (lanes 0) are clear as expected. Bacterial and DNA control samples were done for all selection rounds but can only be seen on gel 7.6a in lanes 1-3 and on gel 7.6c in lanes 5-7. No amplification was seen in the bacterial control samples. The DNA control sample on gel 7.6c in lane 7 has a faint band. The samples were transferred into new fresh tubes after the incubation in order to avoid collecting the aptamers that are binding the tube wall. Even though this step was done; some of the molecules have stayed in the solution where no bacterial cells were added (DNA control).

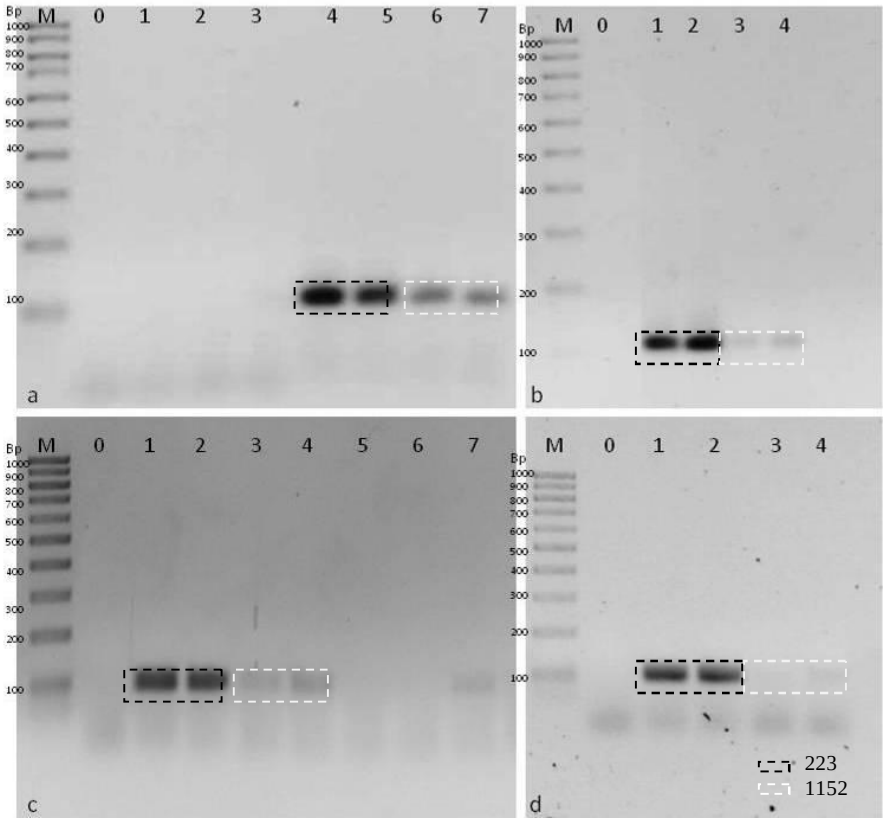


Figure 7.6 Aptamer pools 6 (a), 7 (b), 8 (c) and 9 (d) for *S. typhimurium* 223 (black boxes) and *S. enteritidis* 1152 (white boxes) on agarose gel (2%). Lane M on the gels is a PCR Sizer 100bp DNA Ladder and in lanes 0 is the PCR control. Bacterial control samples are in lanes 1 and 2 on gel a and in lanes 5 and 6 on gel c. The DNA control samples are in lane 3 (a) and in lane 7 on gel c.

7.3.2. Fluorimetric detection of the pathogen binding aptamer pools

The aptamer pools were fluorescent (FAM) labelled (2.3.7) and the binding was detected by fluorimetry. Instead of measuring the DNA concentration of the

aptamers, in this study, the fluorescence values of the aptamers were measured before the aptamers were added to the bacterial cells.

Aptamer pool 9 was previously selected for *E. coli* K12 (3.3.2) and in this study aptamers were selected against *E. coli* 496, *E. coli* 497, *L. monocytogenes* 490, *S. typhimurium* 223 and *S. enteritidis* 1152 and the PCR products were separated on agarose gels. The binding of the aptamers was tested with the previously developed fluorimetry detection assay (4.2.1.3). The binding of the *E. coli* K12 aptamers to their target has previously been demonstrated (3.3.4; 4.3.1) and this aptamer pool was used as a positive control in this experiment.

The fluorescence values of the pathogen samples are shown in Table 7.1. Two samples, as seen in agarose gel images (Figure 7.1-7.6), of each bacterial strain were performed in duplicate. Only one positive control (*E. coli* K12) was performed. The fluorescence values of the aptamer pools before the binding reaction can be seen in row 0. After the incubation with the bacterial cell the samples were centrifuged and washed followed by the fluorescence measurement of the supernatants (1^{st} wash, 2^{nd} wash and 3^{rd} wash). It can be seen that the values are decreasing after each wash. This indicates there are less unbound aptamers in the solution. After the washes the samples were resuspended into the buffer and the fluorescence was measured (Samples) (Table 7.1). The results show that no fluorescence can be detected for *E. coli* 496, *L. monocytogenes* 490 and *S. enteritidis* 1152 while some binding can be detected for *E. coli* O157 497 and *S. typhimurium* 223. It can be seen that the fluorescence values of the pathogen aptamers (*E. coli* 496 and 497, *L. monocytogenes* 490, *Salmonella* 223 and 1152) are much lower than the values of the *E. coli* K12 binding aptamers. The higher the fluorescence the more aptamers have bound. These results indicate there is not much aptamer binding to the pathogen strains. The fluorescence values measured after the 1^{st} wash are very high. When compared to the values in row 0, it can be seen that the most of the aptamers have not bound to the bacterial cells and were washed off. It is possible that there is not enough binding sites on the bacterial cell surface or the aptamers anneal back together to their complementary strand and therefore are not able to form the structure that is needed for their binding to the target. This has previously been discussed more extensively (3.3.2).

Table 7.1 Fluorescence values for *E. coli* K12 (n=4), *E. coli* 496, *E. coli* 497, *L. monocytogenes* 490, *S. typhimurium* 223 and *S. enteritidis* 1152 (n=2).The values are corrected for background.

Fluorescence (495nm, Em 520)	***E. coli* K12**	***E. coli* 496**		***E. coli* 497**		***L. monocytogenes* 490**		***S. typhimurium* 223**		***S. enteritidis* 1152**	
		1	*2*	*1*	*2*	*1*	*2*	*1*	*2*	*1*	*2*
0	2900	2400	2500	2200	2400	400	400	1300	1300	439	539
1st wash	2450	2200	2200	2000	2100	170	240	840	970	170	244
2nd wash	56	90	70	73	82	31	21	161	210	31	21
3rd wash	11	6	7	9	6	3	1	17	18	3	1
Samples	**20**	**0**	**0**	**1.5**	**0.5**	**0**	**0**	**1.5**	**0**	**0**	**0**

The difference between the fluorescence values (row 0) between different samples is also noticeable (Table 7.1). This is mainly caused by the variable concentrations of the aptamers that were mixed with the cells. The difference can already be seen on the agarose gel images where the intensity of the bands varies. For example *S. enteritidis* 1152 aptamers did not yield to a strong PCR product (Figure 7.6) and therefore the fluorescence is also very small compared to *S. typhimurium* 223 aptamers. *S. typhimurium* 223 aptamers also have a strong band on a gel (Figure 7.6c) and the fluorescence is much higher in purified aptamers (Table 7.1, row 0). The aptamer concentrations could have been optimised to this experiment by producing more aptamers by PCR. This would have required several PCR reactions as in a normal binding reaction, when the aptamer concentration is higher, eight PCR reactions were needed. Two aptamer pools were decided to be chosen for further experiments because of the limited time and limited sample handling resources. The aptamer pool 9 against *E. coli* O157 497 and the aptamer pool 9 against *S. typhimurium* 223 were shown to have the best binding properties according to the results presented here and were therefore chosen for further experiments. These aptamers also shown strongest bands on agarose gel images (Figure 7.1 and 7.6) and can therefore be considered to be the best candidates.

Aptamer selection against non-pathogenic *E. coli* was done in a different laboratory than the experiments in this study. It was noticed that the temperature affected to the binding of the *E. coli* K12 aptamers. The ambient temperature in different laboratories, where the experiments were carried out, was different. The *E. coli* K12

selection and the detection (Chapter 3-5) were done in the laboratory where the ambient temperature was 25°C or more when the ambient temperature for the pathogen aptamers was about 22 °C or less. The temperature of the binding reaction was then optimised to 25°C. It was also noticed that the bacterial growth medium affected the aptamer binding. When the *E. coli* K12 bacterial cells were grown in a different growth medium (CASO) than the one when the aptamers were selected (Nutrient broth), the binding was different. The growth media differ in terms of their nutritional composition (CASO is a richer medium with glucose) which in turn may affect the biomolecules present on the bacterial cell surface. It has also been considered that the binding of the aptamer pools should be controlled after each round of selection. It has been demonstrated that sometimes the binding affinity of the aptamers might decrease when the selection has been repeated too many times (Hamula *et al.*, 2008). In the study of Hamula *et al.* (2008) the aptamers were selected to bind to whole bacterial cells and the aptamer pool showed increasing affinity to its target until aptamer pool 7. The affinity decreased dramatically after the eighth round of selection.

7.3.3. Cloning of the aptamers

7.3.3.1. Ligation and transformation

The aptamer pool 9 against *E. coli* O157 and the aptamer pool 9 against *S. typhimurium* 223 were shown to have the best binding properties and were therefore chosen for further experiments. These pools of aptamers with unknown sequences were cloned and sequenced to determine the nucleotide sequence. Aptamer pools 9 were amplified by PCR and cloned with pGEM-T Easy vector and JM109 competent cells. The colonies on indicator plates, containing ampicillin, X-Gal and IPTG were counted and a number of positive (white) and negative (blue) colonies is presented in Table 7.2. The colonies are only growing on the plates due to the ampicillin resistance of the bacteria that has been gained by a successful ligation of the insert into the vector. The background is representing the number of bacteria without the vector growing on the plate and the positive control sample is representing a successful transformation into the competent cells. For both pathogen strains, six white colonies were selected and streaked on new selective plates. After an overnight incubation on selective plates all of these colonies that were white on the first plate were expressing the blue colour (Cl1s-Cl6s, Cl1e, Cl3e-Cl6e), except one sample

that remained white (Cl2e). Sometimes the blue colour may be expressed even though the insert has been ligated into the vector, as previously discussed (5.3.1.1).

Table 7.2 Cloned colonies of *E. coli* O157 and *S. typhimurium* 223 on indicator plates.

Insert:Vector molar ratio		**Blue (negative)**	**White (positive)**	**Total**
Background		8	-	8
Positive control		3	47	50
***E. coli* O157**	**1:4**	20	5	25
	1:2	∞	∞	∞
	1:1	241	18	259
S. typhimurium	**1:4**	95	26	119
	1:2	300	64	364
	1:1	141	18	159

7.3.3.2. PCR analysis for positive colonies

It was described above that some colonies without the vector and the insert may be growing on the indicator plates. It was also previously demonstrated that the colour selection is not reliable way of selecting the positive colonies (5.3.1.2). The colonies from the selective plates were therefore analysed by PCR to see if the insertion of the aptamers into the vector has been successful. Due the use of the aptamer primers PR1 and PR2, only the colonies with the aptamer sequences and the primer binding sites are amplified in PCR which results in a 100 bp PCR product. The agarose gel of the PCR products is shown in Figure 7.7. On the gel the aptamers against *S. typhimurium* 223 are in the lanes Cl1s-Cl6s and against *E. coli* O157 497 in the lanes Cl1e-Cl6e. All of the samples have a 100 bp bands on the gel except sample Cl1e. This result indicates that all of the colonies having the 100 bp band have the aptamer insert in their plasmid. Sample Cl1e was not amplified in PCR and therefore this colony did not contain the plasmid vector with an aptamer ligated in it. The positive colonies (Cl1s-Cl6s and Cl2e-Cl6e) were selected for the further analysis. On the gel in lane 0 is the PCR control and in lane N is the negative control. Both samples are clear as expected. The negative control was amplified by using a colony from cloning ligation control. This colony contained a control insert that does not have a binding site for the aptamer primers PR1 and PR2 and therefore was not expected to produce any PCR products.

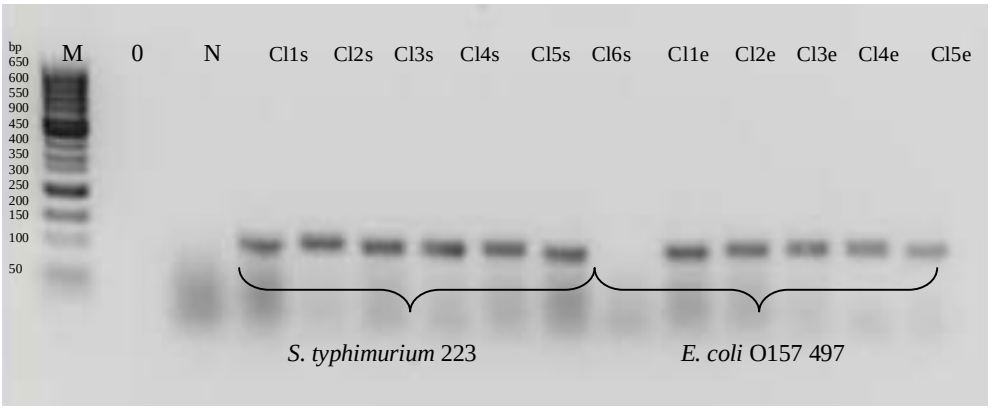


Figure 7.7 2 % Agarose gel with the PCR Spermix HiFi analysis for positive colonies. Lane M on the gel contains PCR MiniSizer 50bp DNA Ladder. In lanes Cl1s-Cl6s are the cloned colonies for *S. typhimurium* aptamers and in lanes Cl1e-Cl6e are the cloned colonies for *E. coli* aptamers. In lane 0 is a PCR control sample and N is negative control.

7.3.4. Sequencing of cloned vectors and aptamer analysis

The cloned aptamer sequences against pathogenic *E. coli* O157 497 (Cl2e-Cl6e) and *S. typhimurium* 223 (Cl1s-Cl6s) were further analysed in order to obtain the DNA sequence. The positive colonies were transferred into a broth and incubated overnight. The plasmid DNA was purified from the overnight culture and the plasmid quality was tested by separating the samples on an agarose gel. In Figure 7.8 the agarose gel image with the plasmid vectors is shown. It can be seen in the figure that all the other bands are the same size except the band in lane Cl2e. This band is larger in size than the other bands and has also a faint band just above the dark band. Interestingly, this sample was the only white colony on the indicator plates after the second day of incubation while the other colonies were expressing the blue colour. The size of the samples (Cl1s-Cl6s and Cl3e-Cl6e) seems to be under 2000 bp when compared to the ladder in lane M. This is not the size that was expected since the plasmid is 3015 bp (+ 100 bp aptamer). The reason for this is the migration of the circular plasmid on the gel. It has generally been recognised that a circular plasmid migrates more rapidly on agarose gel than a linearised plasmid. Therefore the molecular weight marker used in this experiment is not suitable and another gel with another molecular weight marker would have given a better result. It is possible that a less concentrated agarose gel would have also given a better image of the circular plasmids on gel. Four samples for each strain (Cl2e-Cl5e and Cl1s-Cl4s) were sequenced.

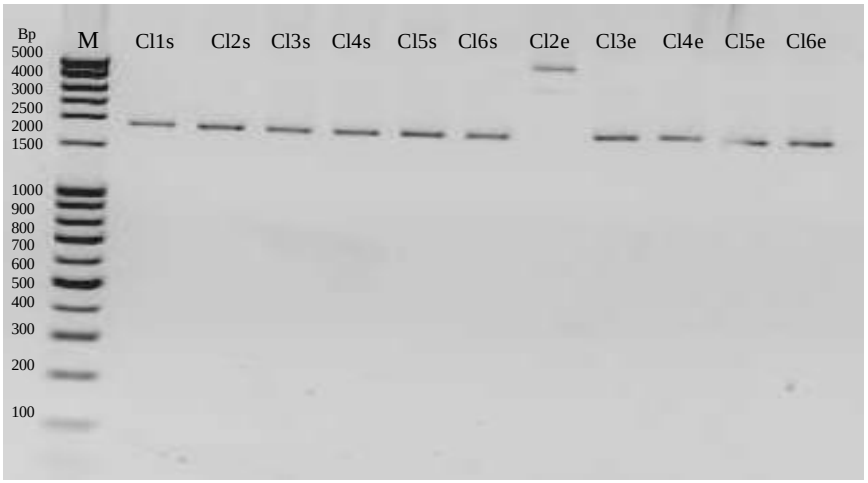


Figure 7.8 2 % Agarose gel with the purified plasmid vectors. Lane M on the gel contains FullRanger 100bp DNA Ladder. In lanes Cl1s-Cl6s are the positive clones for *S. typhimurium* aptamers and in lanes Cl2e-Cl6e are the positive clones for *E. coli* aptamers.

Four samples for both *E. coli* O157 (Cl2e-Cl5e) and *S. typhimurium* (Cl1s-Cl4s) were selected for sequencing. The plasmid DNA was sequenced using the sequencing primers T7 and Sp6r. The aptamer sequences were analysed using the aptamer primers PR1 and PR2 and by finding the matching sequences. The sequencing was successful for all of the samples that had a band in the level of 2000 bp compared to the molecular weight marker in Figure 7.8. The sample Cl2e that looked different on the gel and was the only sample expressing the blue colour on the second indicator plate failed the sequencing. No results were obtained from this sequencing reaction. It is possible the plasmid was damaged in this sample as two bands can be seen on the gel image (Figure 7.8). Most likely the colony growing on the plate did contain the plasmid DNA with the insert aptamer that was used for cloning because the PCR analysis resulted in positive result (Figure 7.7). The most common reasons for the failure in sequencing are insufficient or poor quality template or primer. It is also possible that the primer binding site is deleted or mutated (Eurofins MWG Operon DNA sequencing Result Guide, 2011). The nucleotide sequences of the successfully sequenced aptamers are presented in Table 7.3.

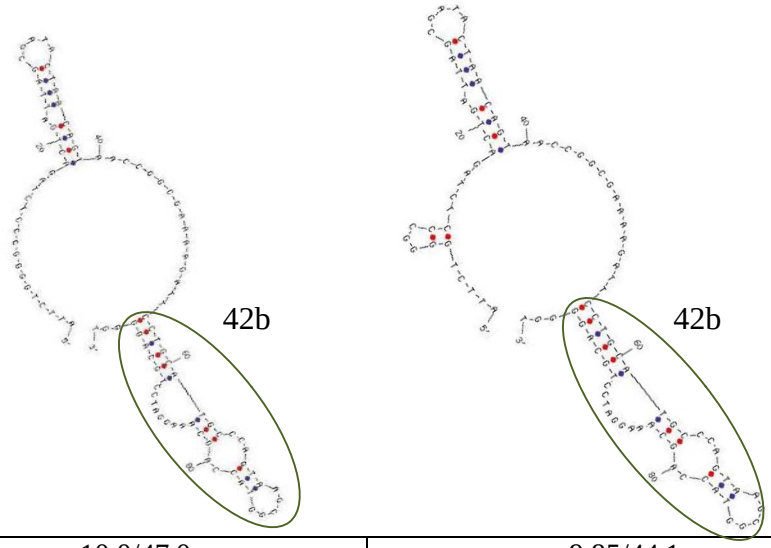
Table 7.3 A list of cloned *E. coli* O157 and *S. typhimurium* binding aptamers and their sequences.

Sample	Size/ Bases	Primer	Sequence
Cl3-Apt497 (Cl3e)	100	T7	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*AACAGTAACCGGCGAAA AGATTCCTGCATGCCCAGTAAGC*GGTACCAGCAAAGGATCCTGCAGGGGT
	100	Sp6r	ACCCCTGCAGGATCCTTTGCTGGTACC*GCTTACTGGGCATGCAGGAATCTT TTCGCCGGTTACTGTT*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
Cl4-Apt497 (Cl4e)	100	T7	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*ACTATCCTCCCCCCTTAGC CATAAATTACGGAGCGGATGA*GGTACCAGCAAAGGATCCTGCAGGGGT
	100	Sp6r	ACCCCTGCAGGATCCTTTGCTGGTACC*TCATCCGCTCCGTAATTTATGGCTA AGGGGGGAGGATAGT*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
Cl5-Apt497 (Cl5e)	100	T7	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*CTCCCACCTACGCCTTAAC TTTTCCAACATCCTCCATCGC*GGTACCAGCAAAGGATCCTGCAGGGGT
	100	Sp6r	ACCCCTGCAGGATCCTTTGCTGGTACC*GCGATGGAGGATGTTGGAAAAGT TAAGGCGTAGGTGGGAG*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
Cl1-Apt223 (Cl1s)	100	T7	ACCCCTGCAGGATCCTTTGCTGGTACC*TAGAAGCCGGCCGTAGAGGAGGA AAGGATGATAACTTGCT*AGTATCGCTAATCAGTCTAGATGGCCCCAGAAT
	100	Sp6r	ATTCTGGGGCCATCTAGACTGATTAGCGATACT*AGCAAGTTATCATCCTTTC CTCCTCTACGGCCGGCTTCTA*GGTACCAGCAAAGGATCCTGCAGGGGT
Cl2-Apt223 (Cl2s)	100	T7	ACCCCTGCAGGATCCTTTGCTGGTACC*AGGGAAATCGTAGTTGATTACGAT TATGACGTAGTGTACC*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
	100	Sp6r	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*GGTACACTACGTCATAAT CGTAATCAACTACGATTTCCCT*GGTACCAGCAAAGGATCCTGCAGGGGT
Cl3-Apt223 (Cl3s)	100	T7	ACCCCTGCAGGATCCTTTGCTGGTACC*GTAGACTATCATTTTCACTAACGTA CAGGGAGCGGGGCTG*AGTATCGCTAATCAGTCTAGAGGGCCCCAGTAT
	100	Sp6r	ATACTGGGGCCCTCTAGACTGATTAGCGATACT*CAGCCCCGCTCCCTGTAC GTTAGTGAAAATGATAGTCTAC*GGTACCAGCAAAGGATCCTGCAGGGGT
Cl4-Apt223 (Cl4s)	100	T7	ACCCCTGCAGGATCCTTTGCTGGTACC*TAAGACTTGGAGTAAGACAGTGA CAGGTATATTGGTTGTG*AGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT
	100	Sp6r	ATTCTGGGGCCCTCTAGACTGATTAGCGATACT*CACAACCAATATACCTGT CACTGTCTTACTCCAAGTCTTA*GGTACCAGCAAAGGATCCTGCAGGGGT

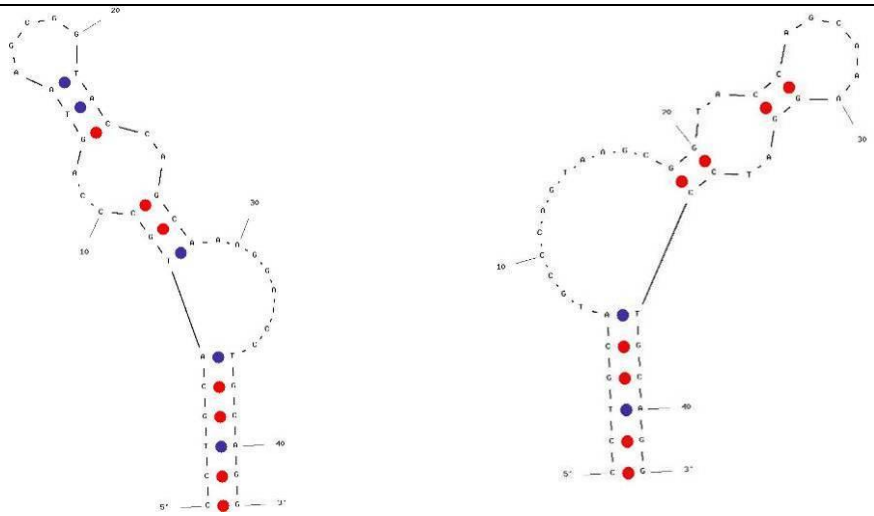
In order to synthesise the aptamers the number of nucleotides had to be reduced (James, 2000). The aptamer sequences (rows T7 in Table 7.3) Cl3-Apt497, Cl4-Apt497, Cl5-Apt497, Cl1-Apt223, Cl2-Apt223, Cl3-Apt223 and Cl4-Apt223 were further analysed using the UNAFold program that gives aptamer secondary structures most likely to be formed in buffer conditions. The structures are presented

in Figure 7.9 (Cl3-Apt497), Figure 7.10 (Cl4-Apt497), Figure 7.11 (Cl5-Apt497), Figure 7.12 (Cl1-Apt223), Figure 7.13 (Cl2-Apt223), Figure 7.14 (Cl3-Apt223) and Figure 7.15 (Cl4-Apt223). Two structures for 100 nt sequences are presented in the first line and the aptamer sequences that were cut off from these 100 nt sequences are presented below. The sequences with reduced lengths are marked by circle or circles in the pictures and the strongest isolated sequences with their secondary structures are shown for each sample below. For aptamer 5Apt497 (Figure 7.11) only one structure is shown because this is the only way the sequence can fold according to UNAFold. The ΔG was calculated by the UNAFold and it is defined as a change in Gibbs energy when the system undergoes a thermodynamic change. The tighter the hairpin structure of the aptamer is, the stronger binding has been observed (Hamula *et al.*, 2008; 5.3.3). Generally two different sequences were isolated from the 100 nt sequence but in some sequences only one sequence could be isolated (Cl3-Apt497, Cl2-Apt223, Cl4-Apt223).

Cl3-Apt497	ATTCTGGGGCCCTCTAGACTGATTAGCGATACTAACAGTAACCGGCGAAAAGATTCCTGCATGCCCAGTAAGCGGTACCAGCAAAGGATCCTGCAGGGGT



ΔG/Tm(°C)	-10.0/47.0	-9.85/44.1
1Apt497 42 nt	*CCTGCATGCCCAGTAAGCGGTACCAGCAAAGGATCCTGCAGG*	



ΔG/Tm(°C)	-5.12/45.6	-3.27/40.2

Figure 7.9 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25°C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *E. coli* O157 497 binding nucleotide sequence (Cl3-Apt497). Aptamer 1Apt497 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Blue and red dots represent the base-pair interactions.

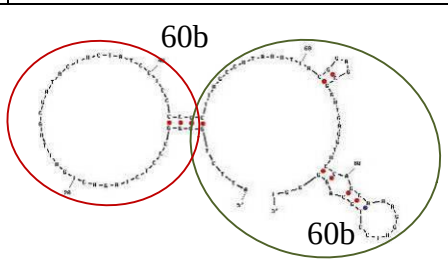
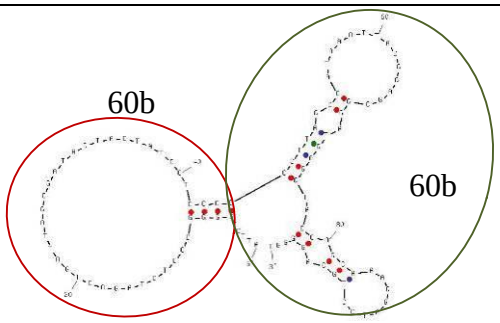
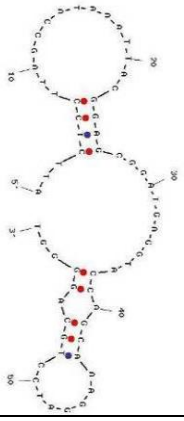
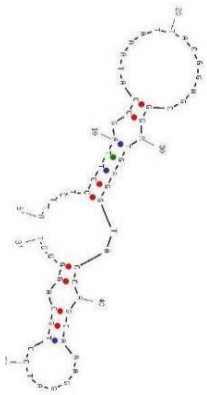
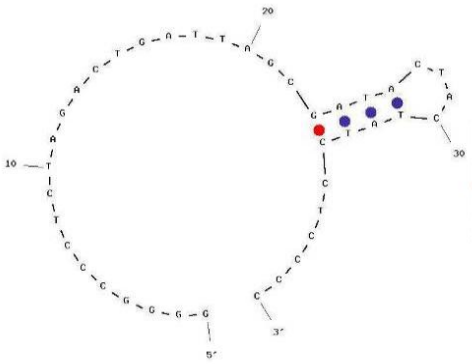
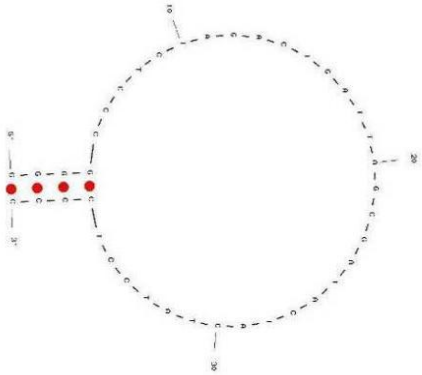
Cl4-Apt497	ATTCTGGGGCCCTCTAGACTGATTAGCGATACTACTATCCTCCCCCCTTAGCCATAAATTACGG AGCGGATGAGGTACCAGCAAAGGATCCTGCAGGGGT	
		
ΔG/Tm(°C)	-4.58/39.9	-4.54/38.6
2Apt497 60 nt	ATTCT *CCTTAGCCATAAATTACGGAGCGGATGAGGTACCAGCAAAGGATCCTGCAGGGGT	
		
ΔG/Tm(°C)	-4.06/43.2	-3.99/41.5
3Ap223 60 nt	*GGGGCCCTCTAGACTGATTAGCGATACTACTATCCTCCCC*	
		
ΔG/Tm(°C)	-0.56/30.7	-0.49/30.4

Figure 7.10 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25 °C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *E. coli* O157 497 binding nucleotide sequence (Cl4-Apt497). Aptamers 2Apt497 and 3Apt223 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Blue and red dots represent the base-pair interactions.

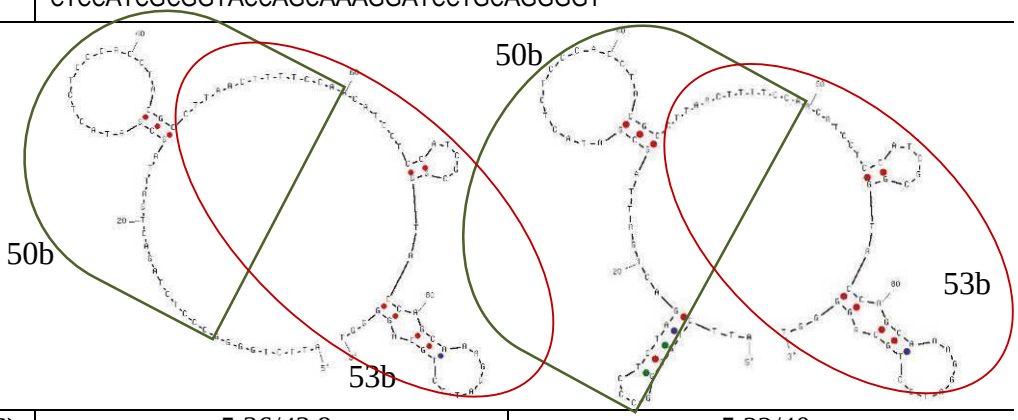
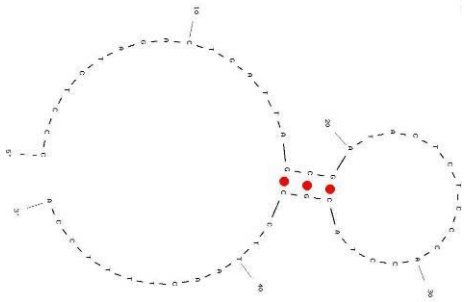
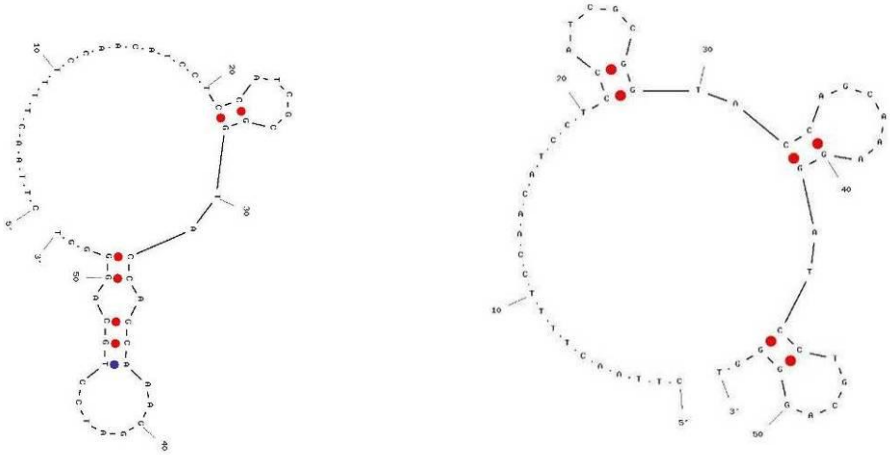
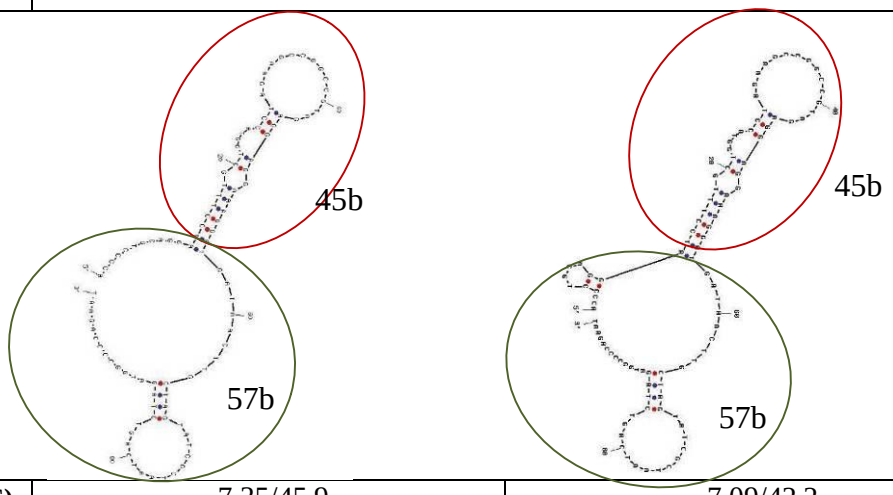
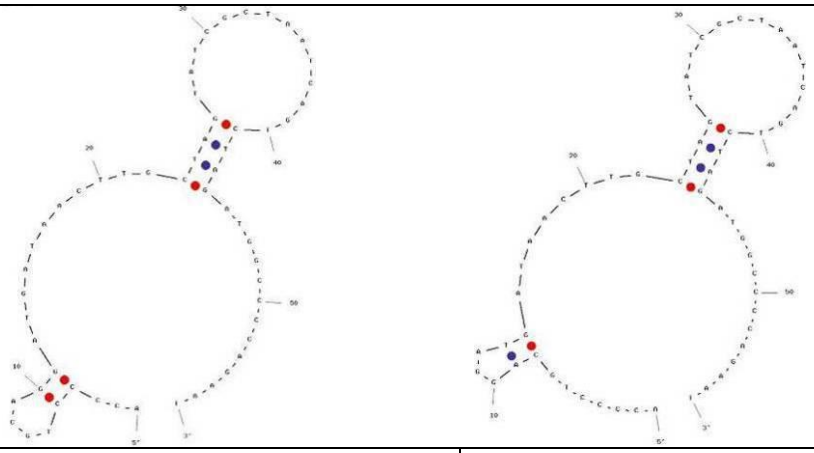
Cl5-Apt497	ATTCTGGGGCCCTCTAGACTGATTAGCGATACTCTCCCACCTACGCCTTAACTTTTCCAACATC CTCCATCGCGGTACCAGCAAAGGATCCTGCAGGGGT	
		
ΔG/Tm(°C)	-5.36/43.8	-5.22/40
4Apt497 50 nt	*CCCTCTAGACTGATTAGCGATACTCTCCCACCTACGCCTTAACTTTTCCA*	
		
ΔG/Tm(°C)	-2.28/47.4	
5Apt497 53 nt	*CTTAACTTTTCCAACATCCTCCATCGCGGTACCAGCAAAGGATCCTGCAGGGGT	
		
ΔG/Tm(°C)	-3.08/41.9	-2.65/38.9

Figure 7.11 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25°C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *E. coli* O157 497 binding nucleotide sequence (Cl5-Apt497). Aptamers 4Apt497 and 5Apt497 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Blue and red dots represent the base-pair interactions.

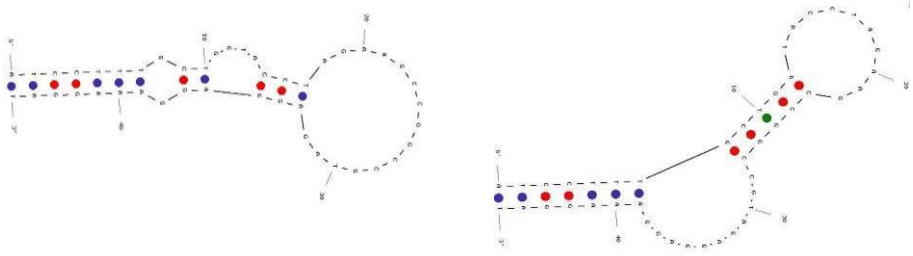
Cl1-Apt223	ACCCCTGCAGGATCCTTTGCTGGTACCTAGAAGCCGGCCGTAGAGGAGGAAAGGATGATAA CTTGCTAGTATCGCTAATCAGTCTAGATGGCCCCAGAAT



ΔG/Tm(°C)	-7.35/45.9	-7.09/42.2
1Apt223 57 nt	ACCCCTGCAGGA*TGATAACTTGCTAGTATCGCTAATCAGTCTAGATGGCCCCAGAAT	



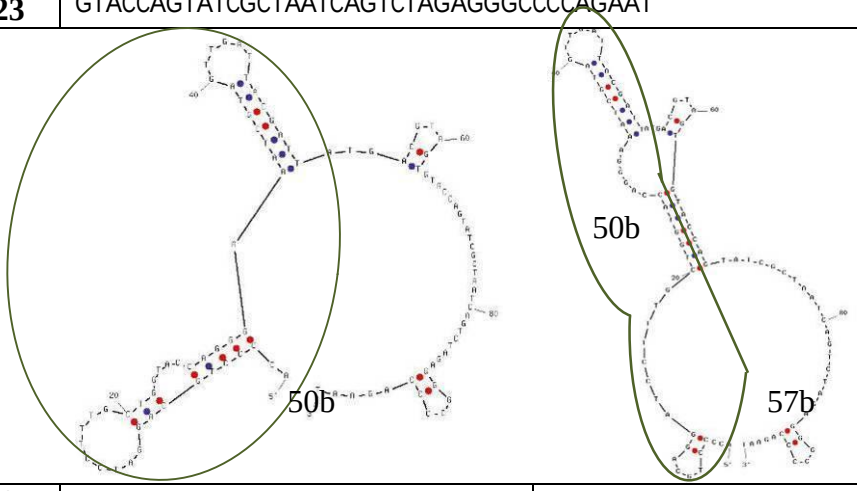
ΔG/Tm(°C)	-2.74/40.2	-2.28/39.1
2Ap223 45 nt	*ATCCTTTGCTGGTACCTAGAAGCCGGCCGTAGAGGAGGAAAGGAT*	



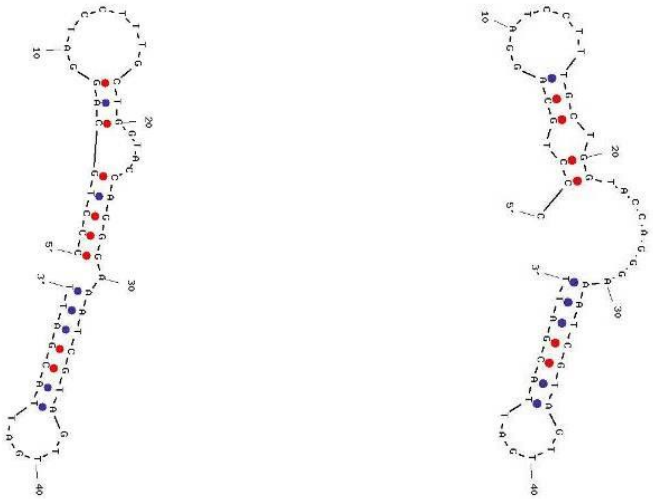
ΔG/Tm(°C)	-4.76/45.7	-3.90/42.1

Figure 7.12 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25°C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *S. typhimurium* 223 binding nucleotide sequence (Cl1-Apt223). Aptamers 1Apt223 and 2Apt223 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Blue and red dots represent the base-pair interactions.

Cl2-Apt223	ACCCCTGCAGGATCCTTTGCTGGTACCAGGGAAATCGTAGTTGATTACGATTATGACGTAGT GTACCAGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT



ΔG/Tm(°C)	-10.7/43.4	-10.68/43.6
3Apt223 50 nt	*CCCTGCAGGATCCTTTGCTGGTACCAGGGAAATCGTAGTTGATTACGATT*	



ΔG/Tm(°C)	-8.59/51.2	-8.51/53.3

Figure 7.13 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25°C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *S. typhimurium* 223 binding nucleotide sequence (Cl2-Apt223). Aptamer 3Apt223 has been created by cutting off (*) the possible binding site from the 100 nt sequence. Isolated sequences are circled. Blue and red dots represent the base-pair interactions.

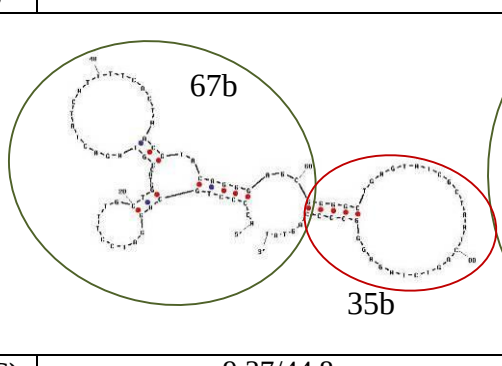
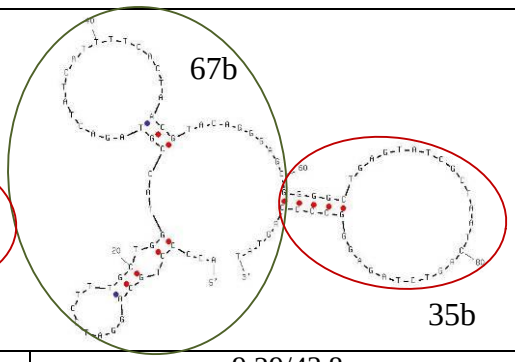
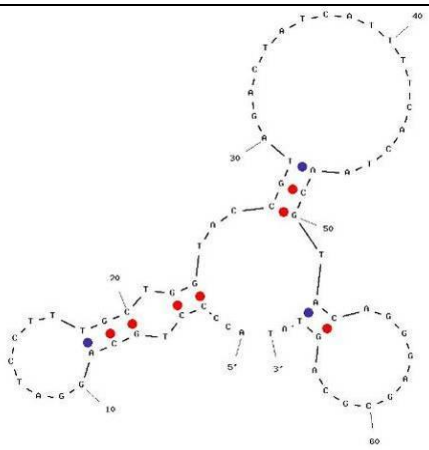
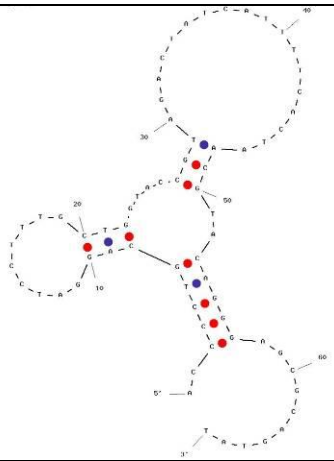
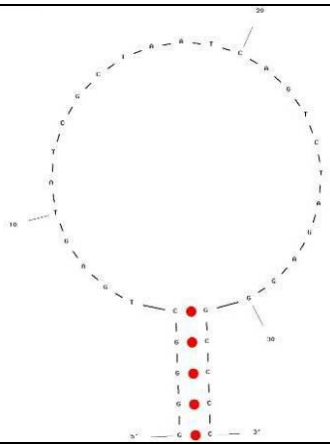
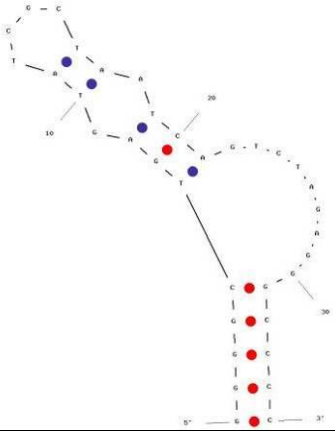
Cl3-Apt223	ACCCCTGCAGGATCCTTTGCTGGTACCGTAGACTATCATTTTCACTAACGTACAGGGAGCGGG GCTGAGTATCGCTAATCAGTCTAGAGGGCCCCAGTAT	
		
ΔG/Tm(°C)	-9.37/44.8	-9.29/43.8
4Apt223 67 nt	ACCCCTGCAGGATCCTTTGCTGGTACCGTAGACTATCATTTTCACTAACGTACAGGGAGCG*C AGTAT	
		
ΔG/Tm(°C)	-3.46/38.4	-4.36/41.3
5Ap223 35 nt	* GGGGCTGAGTATCGCTAATCAGTCTAGAGGGCCCC *	
		
ΔG/Tm(°C)	-3.68/55.5	-3.50/43.6

Figure 7.14 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25°C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *S. typhimurium* 223 binding nucleotide sequence (Cl3-Apt223). Aptamers 4Apt223 and 5Apt223 have been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Blue and red dots represent the base-pair interactions.

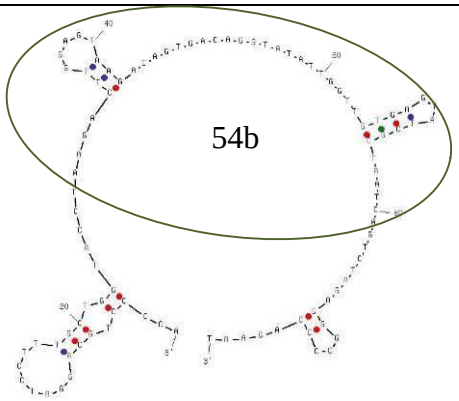
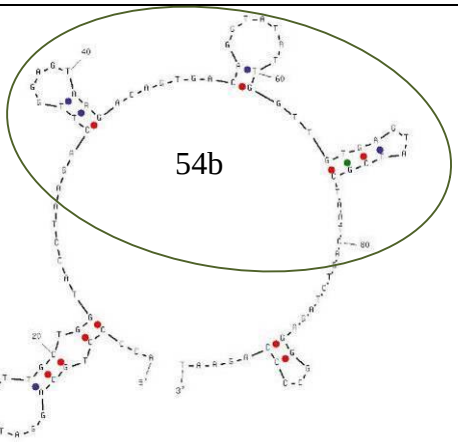
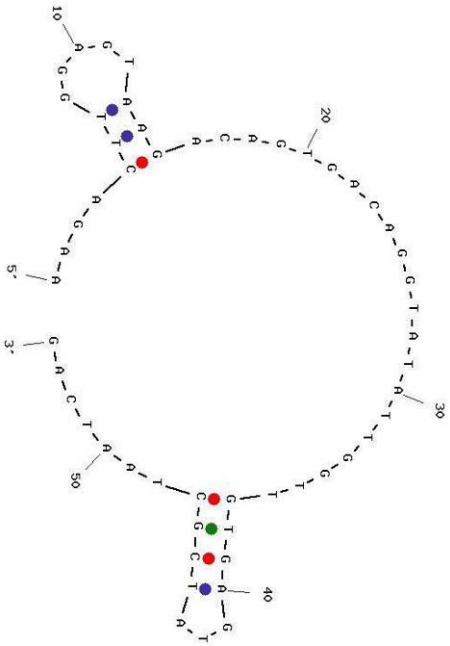
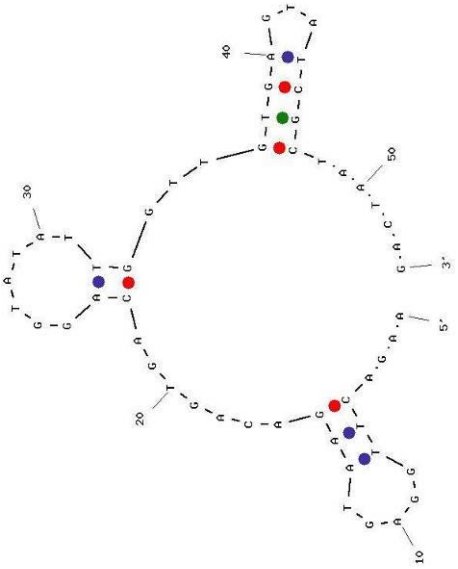
Cl4-Apt223	ACCCCTGCAGGATCCTTTGCTGGTACCTAAGACTTGGAGTAAGACAGTGACAGGTATATTGG TTGTGAGTATCGCTAATCAGTCTAGAGGGCCCCAGAAT	
		
ΔG/Tm(°C)	-6.63/42.8	-6.20/38.9
6Apt223 54 nt	*AAGACTTGGAGTAAGACAGTGACAGGTATATTGGTTGTGAGTATCGCTAATCAG*	
		
ΔG/Tm(°C)	-3.0/43.3	-2.58/35.8

Figure 7.15 Predicted aptamer secondary structures (OligoAnalyzer 3.1, UNAFold) at 25°C (NaCl 100 mM, MgCl 1 mM). The two strongest secondary structures for *S. typhimurium* 223 binding nucleotide sequence (Cl4-Apt223). Aptamer 6Apt223 has been created by cutting off (*) the possible binding sites from the 100 nt sequence. Isolated sequences are circled. Blue and red dots represent the base-pair interactions.

Changing a nucleotide or several nucleotides within the aptamer sequence may enable production of more molecules with target specificity (Bini, *et al*., 2011). It has previously been recognised that the aptamer structures can be rationally engineered with modification or edition (insertion/deletion) of nonconserved regions (Zhang *et al.*, 2008). Changing one or several nucleotides might also produce more stable aptamer structure or it might also lead to a complete change of the aptamer structure. Even a mononucleotide substitution is sufficient to alter the 3D shape of diverse secondary and tertiary structures formed by different sequences (Gening *et al.*, 2001).

The secondary structures as well as energy needed to break the structure (ΔG) were compared and the three strongest aptamers for each pathogenic strain (*S. typhimurium* and *E. coli* O157) were selected and synthesised with FAM-labels. The synthesised aptamers along with their size and ΔG values are listed in Table 7.4. The aptamer 4Apt497 was selected instead of aptamer 5Apt497 that has a ΔG value -3.08 because aptamer 4Apt497 has only one secondary structure (Figure 7.11). These six aptamers were further analysed by testing the binding and the specificity.

Table 7.4 Sequences and ΔG values for the synthesised FAM-labelled aptamers.

Aptamer	**Nt**	**Sequence**	**ΔG**
1Apt497	42	5' FAM-CCTGCATGCCCAGTAAGCGGTACCAGCAAAGGATCCTGCAGG -3'	-5.12
2Apt497	60	5' FAM-ATTCTCCTTAGCCATAAATTACGGAGCGGATGAGGTACCAGCAAAGGATCCTGCAGGGGT-3'	-4.06
4Apt497	50	5' FAM-CCCTCTAGACTGATTAGCGATACTCTCCCACCTACGCCTTAACTTTTCCA -3'	-2.28
2Apt223	45	5'FAM-ATCCTTTGCTGGTACCTAGAAGCCGGCCGTAGAGGAGGAAAGGAT -3'	-4.76
3Apt223	50	5' FAM-CCCTGCAGGATCCTTTGCTGGTACCAGGGAAATCGTAGTTGATTACGATT -3'	-8.59
5Apt223	35	5' FAM-GGGGCTGAGTATCGCTAATCAGTCTAGAGGGCCCC -3'	-3.68

7.3.5. Binding of the cloned aptamers

7.3.5.1. *E. coli* O157 aptamers

Aptamers selected to bind to pathogenic *E. coli* O157 497 were cloned and sequenced. The aptamer sequences (1Apt497, 2Apt497 and 4Apt497) were synthesised with a fluorescent label and the binding was tested against live *E. coli* O157 497 bacterial cells. The aptamers (10, 20 and 50 pmol) were incubated with the bacterial cells in triplicate. The binding was analysed with a fluorimetry analysis and the samples were examined under the fluorescence microscope. The fluorescence values are presented in Figure 7.16. The results show that when the aptamers have been added the fluorescence values are greater. Even though the error bars are large the data analysis showed that the fluorescence is significantly higher in all samples 1Apt497 (F=4.24, P=0.05), 2Apt497 (F=4.5, P=0.03) and 4Apt497 (F=6.47, P=0.02) when the aptamers have been added. According to the results an addition of 20 pmol of aptamers is enough to detect the fluorescence in the samples 1Apt497 (F=10, P=0.03) and 4Apt497 (F=112.5, P=4.5×10^{-4}) since the addition of 50 pmol of aptamer 1Apt497 did not result in significantly higher fluorescence. For aptamer 2Apt497 the addition of 50 pmol is required (F=8.5, P=0.04). These results indicate the aptamers 1Apt497, 2Apt497 and 4Apt497 are binding to *E. coli* O157 497 bacterial cells. The large error bars could be caused by the variable number of the bacterial cells. It is possible that too many bacterial cells have been washed off during the washing steps. This is the first report of the DNA aptamer sequences against *E. coli* O157 (Kärkkäinen *et al.*, 2011a)

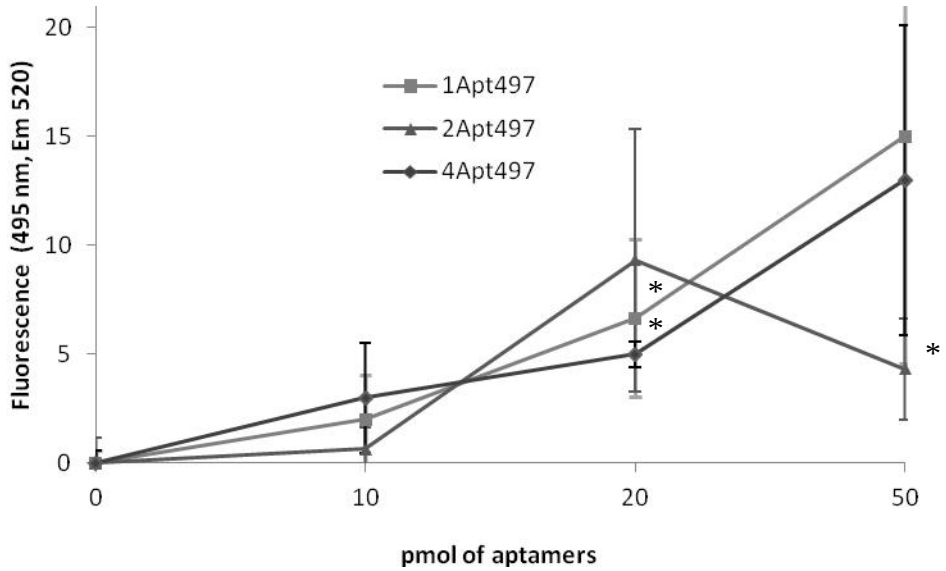


Figure 7.16 FAM-labelled aptamers binding to *E. coli* O157 497 cells. The fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (0.0). *Significant ($P \leq 0.05$).

Samples were visualised under the microscope with a green fluorescence and a visible light. There was no great difference between the images taken of the samples with different aptamer concentrations. The images taken from 20 pmol samples are shown in Figure 7.17 where the fluorescence microscope images are on the left hand side and the visible light images on the right hand side. The bacterial cells with fluorescent labelled aptamers bound to them can be seen on the images as bright green dots. No aptamers were added to the background samples. It can be seen in the images that not many bright dots are visible compared to the background. It was noticeable when looking at the microscope images that there were no bright dots all over the microscope slide. It can be possible that some aptamers have bound to the surface of the bacterial cells but they cannot be detected in the microscope images.

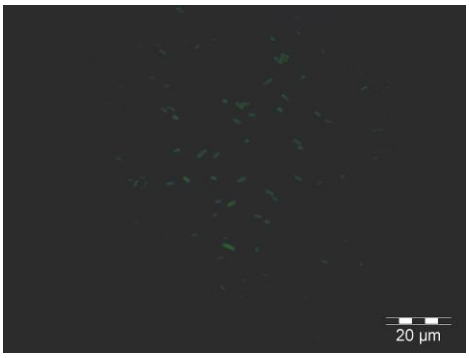
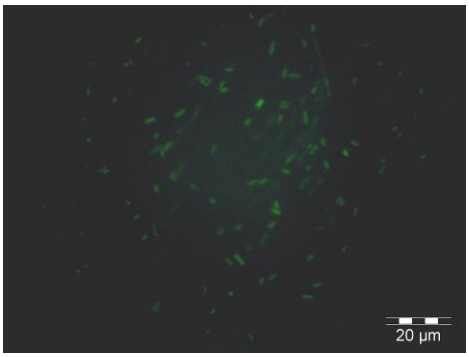
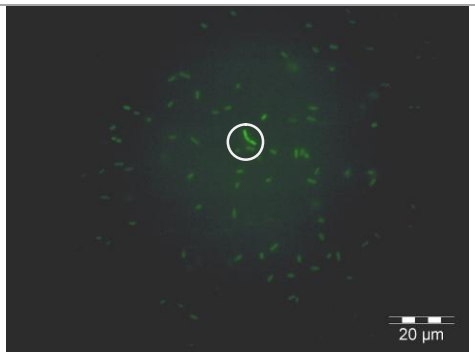
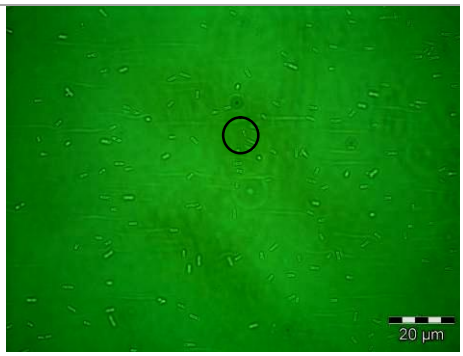
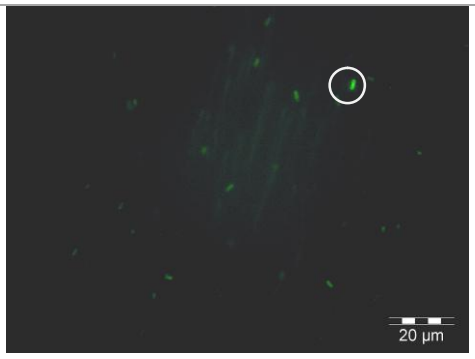
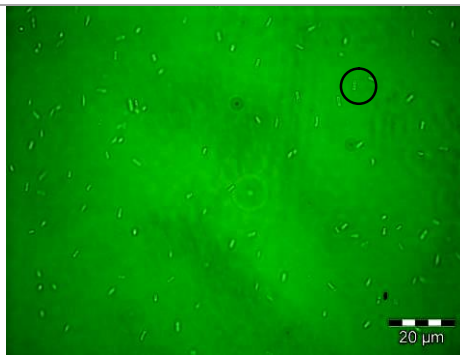
Aptamers (50 pmol)	Fluorescence, green light (495 nm)	Visible light
Background (no aptamers)		
1Apt497		
2Apt497		
4Apt497		

Figure 7.17 Microscopy images of FAM-labelled aptamers 1Apt497, 2Apt497 and 4AptK12 (20 pmol) binding to the surface of *E. coli* O157 497. Images were taken with a fluorescence microscope with 100× magnification with a green (495 nm) and visible light. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

The fluorescent FAM label that is attached to the aptamers is only a small molecule (6-Carboxyfluorescein). If only one aptamer has bound to a bacterial cell it is very likely that this bacterial cell is not visible in the microscope image. Although some bright dots that were not visible in background samples can be detected in the image where the aptamers were added. This result indicates that some aptamer binding can be detected. *E. coli* O157 bacterial cells are relatively small in size and move rapidly on the microscope slide. The movement is mainly due by the capillary action on the microscope slide. This made the visualisation of the bacterial cells challenging and therefore some of the images are not clear. Bright green bacterial cells were often seen when two bacterial cells were connected to each other (dividing cells). This can be seen for example in the fluorescence images of sample 2Apt497 and 4Apt497. In future, in order to get clearer microscope images, the motility of the bacterial cells could be reduced by methyl cellulose solution that increases the viscosity of the solution (Pijper, 1946). It is also possible to fix the bacterial cells on the microscope slide with acetone or some alcohols. The fixation can also be done just simply passing the microscope slide through the flame for a couple of times (Cao *et al.*, 2009).

7.3.5.2. Binding of *E. coli* O157 aptamers to *E. coli* K12

E. coli O157 aptamers were selected from a pool of *E. coli* K12 binding aptamers. The binding of these three cloned aptamers 1Apt497, 2Apt497 and 4Apt497 was tested against *E. coli* K12 by fluorimetry. The aptamers (20 pmol) were incubated with the bacterial cells and the fluorescence was measured. Aptamer 4AptK12 was used as a positive control as the binding of this aptamer has previously been demonstrated (5.3.4.1).

The fluorescence values that are presented in Figure 7.18 were measured for all the samples (1Apt497, 2Apt497, 4Apt497, 4AptK12) and some binding can be detected from each sample. The fluorescence values for all four samples were significantly higher when the aptamers had been added compared to the background fluorescence (1Apt497 F=7.56, P=0.05; 2Apt497 F=39.8, P=8.1×10^{-3}; 4Apt497 F=105.6, P=5.0×10^{-4}; 4AptK12 F=195.9, P=1.5×10^{-4}). It can be seen in the figure that the fluorescence was significantly higher when the aptamer 4AptK12 was incubated with *E. coli* K12 bacterial cells (F=68.25 P=1.5×10^{-5}). This result indicated that the

aptamers selected to bind to *E. coli* O157 497 from a pool of *E. coli* K12 aptamers were also binding to *E. coli* K12, but not as strongly as the *E. coli* K12 binding aptamers. Due to a technical problem with the fluorescence plate reader (as seen in section 5.3.4.2) the fluorescence of the samples was measured with a sensitivity of 75 instead of 50. This means that the fluorescence values from this study cannot be directly compared to the values obtained in previous experiments but the trend is still consistent. Exception from this is Figure 5.12 (5.3.4.2) where the specificity of the individual aptamers was tested. It can be seen that the fluorescence value for aptamer 4AptK12 is much higher here in Figure 7.18 than it was in Figure 5.12 even though a 20 pmol aptamer concentration was used in both experiments. The difference in the fluorescence values may have been caused by the plate reader problem or due to a difference in the number of bacterial cells in the samples. However, there is no need to compare the results from these two experiments to each other as both of these experiments have been corrected for background readings. When looking at the fluorescence values previously measured for *E. coli* K12 aptamer 4AptK12, for example in Figure 5.8, it can be seen that the fluorescence is about 30 units less than the measurement (Figure 7.18) with the sensitivity of 75. This information can be used when comparing the results and in that case the fluorescence measured for the aptamers 1Apt497, 2Apt497 and 4Apt497 are showing more binding against *E. coli* O157 497 (Figure 7.16) than against *E. coli* K12 (Figure 7.18).

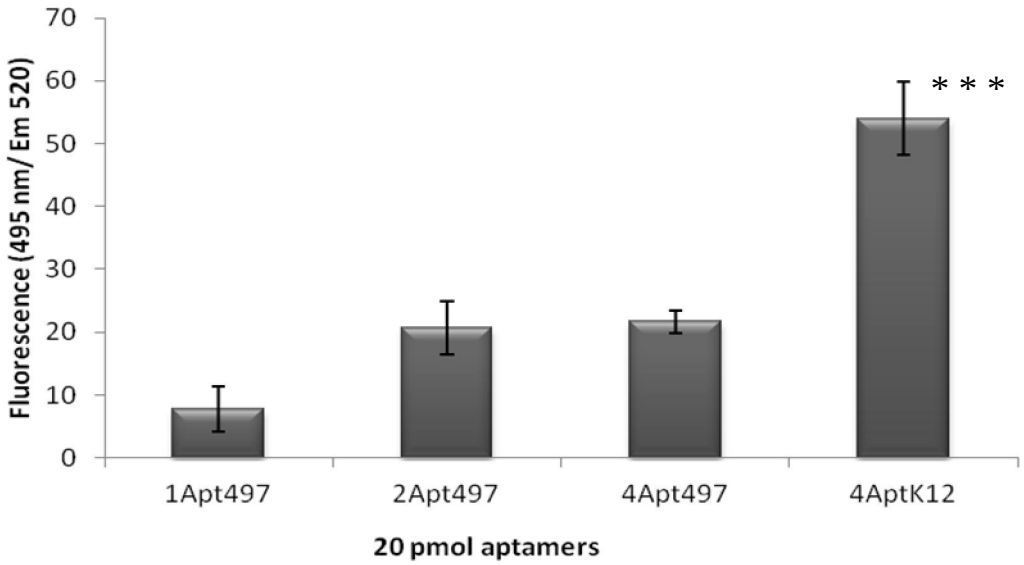


Figure 7.18 FAM-labelled aptamers 1Apt497, 2Apt497, 4Apt497 and 4AptK12 binding to live *E. coli* K12 cells. Fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (35.3). *** very highly significant (P<0,001).

The samples were visualised under the fluorescence microscope with 60× magnification. The microscope images are presented in Figure 7.19. The fluorescence images are on left hand side and the visible light images on right hand side. It can be seen in the images that when the *E. coli* K12 bacteria cells were incubated with the aptamer 4AptK12 (positive control), bright green dots can be seen. Some fluorescent dots can also be seen when *E. coli* K12 was incubated with the pathogen aptamers 1Apt497, 2Apt497 and 4Apt497 but the fluorescence is not as bright in the aptamer 4AptK12 image. This result confirms some binding of the aptamer 1Apt497, 2Apt497 and 4Apt497 to *E. coli* K12 can be detected.

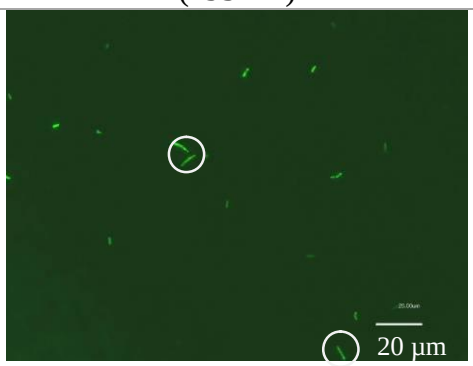
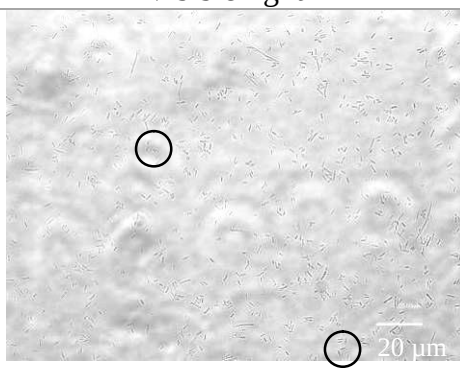
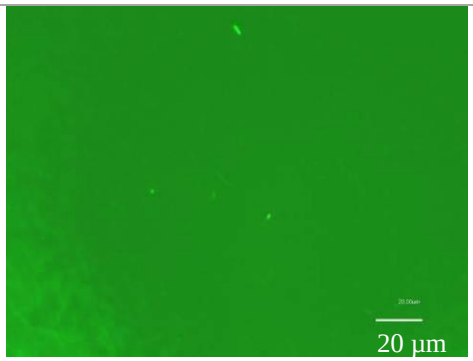
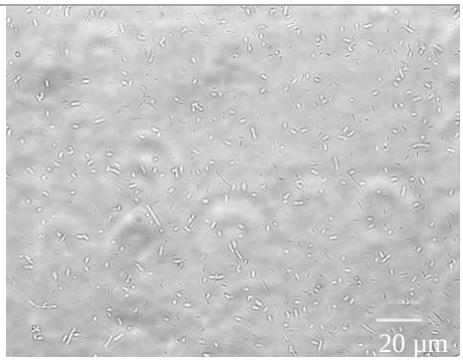
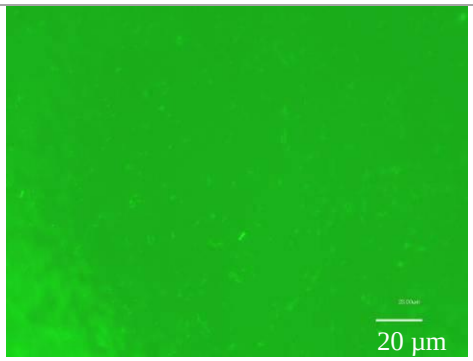
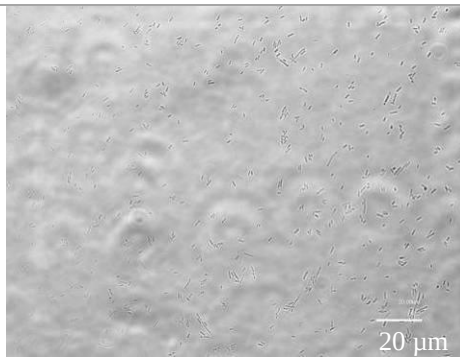
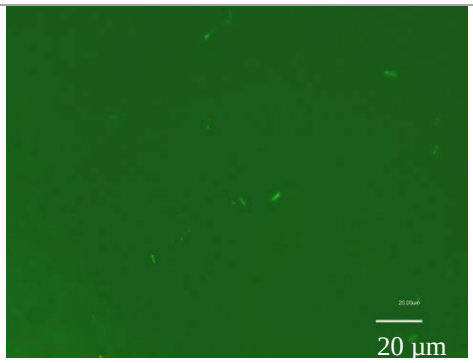
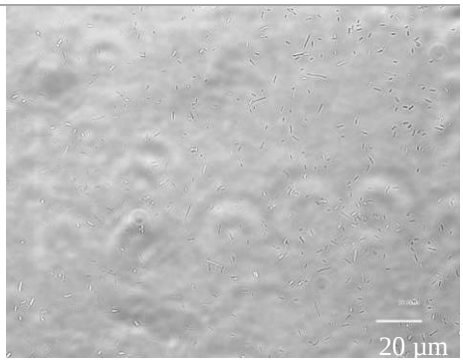
Aptamers (20 pmol)	Fluorescence, green light (495 nm)	Visible light
4AptK12		
1Apt497		
2Apt497		
4Apt497		

Figure 7.19 Microscopy images of FAM-labelled *E. coli* O157 aptamers 1Apt497, 2Apt497 and 4Apt497 (20 pmol) binding to the surface of *E. coli* K12. Images were taken with a fluorescence microscope with 60× magnification with a green (495 nm) and visible light. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

It needs to be considered if the fluorescent microscopy is sensitive enough for detection of the bound aptamers. Aptamers were selected to bind pathogenic bacteria but the microscope pictures did not clearly show the aptamer binding to the bacterial cells. When looking at the fluorescence microscope images taken with the *E. coli* K12 aptamer (Figure 5.9) it can clearly be seen that the fluorescent-labelled aptamers have bound to bacterial cells. The size of the bacterial cells might influence that as previously discussed (7.3.5.1). Another consideration is that is only a few aptamers have bound to the bacterial cell surface, therefore it is likely that those small fluorescence labels cannot be detected under the microscope.

7.3.5.3. *S. typhimurium* binding aptamers

The aptamers that were selected to bind to *S. typhimurium* 223 were cloned and the sequences were synthesised with fluorescent labels. The binding of the FAM-aptamer sequences 2Apt223, 3Apt223 and 5Apt223 was tested against live *S. typhimurium* 223 bacterial cells. The aptamers (10, 20 and 50 pmol) were incubated with the bacterial cells in triplicate and the binding was analysed by fluorimetry. The fluorescence values are presented in Figure 7.20. The results show that the greater the number of added aptamers the higher the fluorescence. The fluorescence is significantly higher in all samples 2Apt223 (F=233.8, P=7.9×10^{-10}), 3Apt223 (F=87.8, P=9.54×10^{-8}) and 5Apt223 (F=23.1, P=4.9×10^{-5}) when the aptamer have been added. The results indicate that the addition of 10 pmol of *S. typhimurium* aptamers is enough to see a significantly higher fluorescence values than when the aptamers have not been added (2Apt223: F=64, P=1.3×10^{-3}; 3Apt223: F=48.9, P=2.2×10^{-3}; 5Apt223: F=25.9, P=7.0×10^{-3}). Aptamers against surface proteins of *S. typhimurium* have previously been selected (Joshi *et al.* 2009) but these three individual sequences are the first ones reported (Kärkkäinen *et al.*, 2011a)

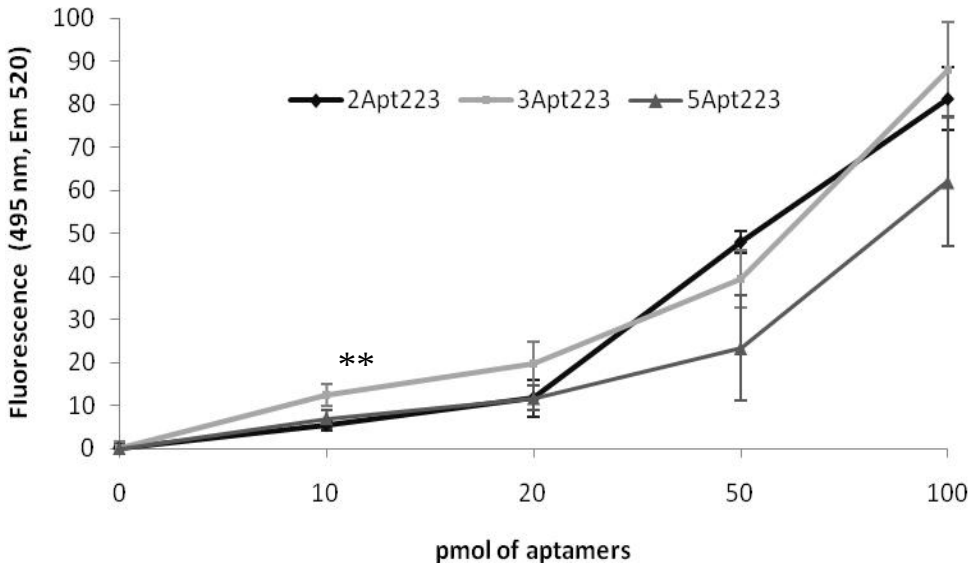


Figure 7.20 FAM-labelled aptamers binding to live *S. typhimurium* 223 cells. Fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (0.0). ** Highly significant (P<0,001).

The samples were visualised under a microscope with a green fluorescent light and a visible light. There was no great difference between the images taken of the samples with different aptamer concentrations. The images for 20 pmol are shown in Figure 7.21. The fluorescence microscope images are on the left hand side and the visible light images are on the right hand side. The bacterial cells with fluorescent labelled aptamers bound to them can be seen on the images as bright green dots. No aptamers were added to the background samples. In the images not many bright green bacterial cells can be seen. It is possible that the aptamers are only binding to some of the bacterial cell or only a small number of aptamers is binding to cell and therefore the bacterial cells cannot be detected in the images.

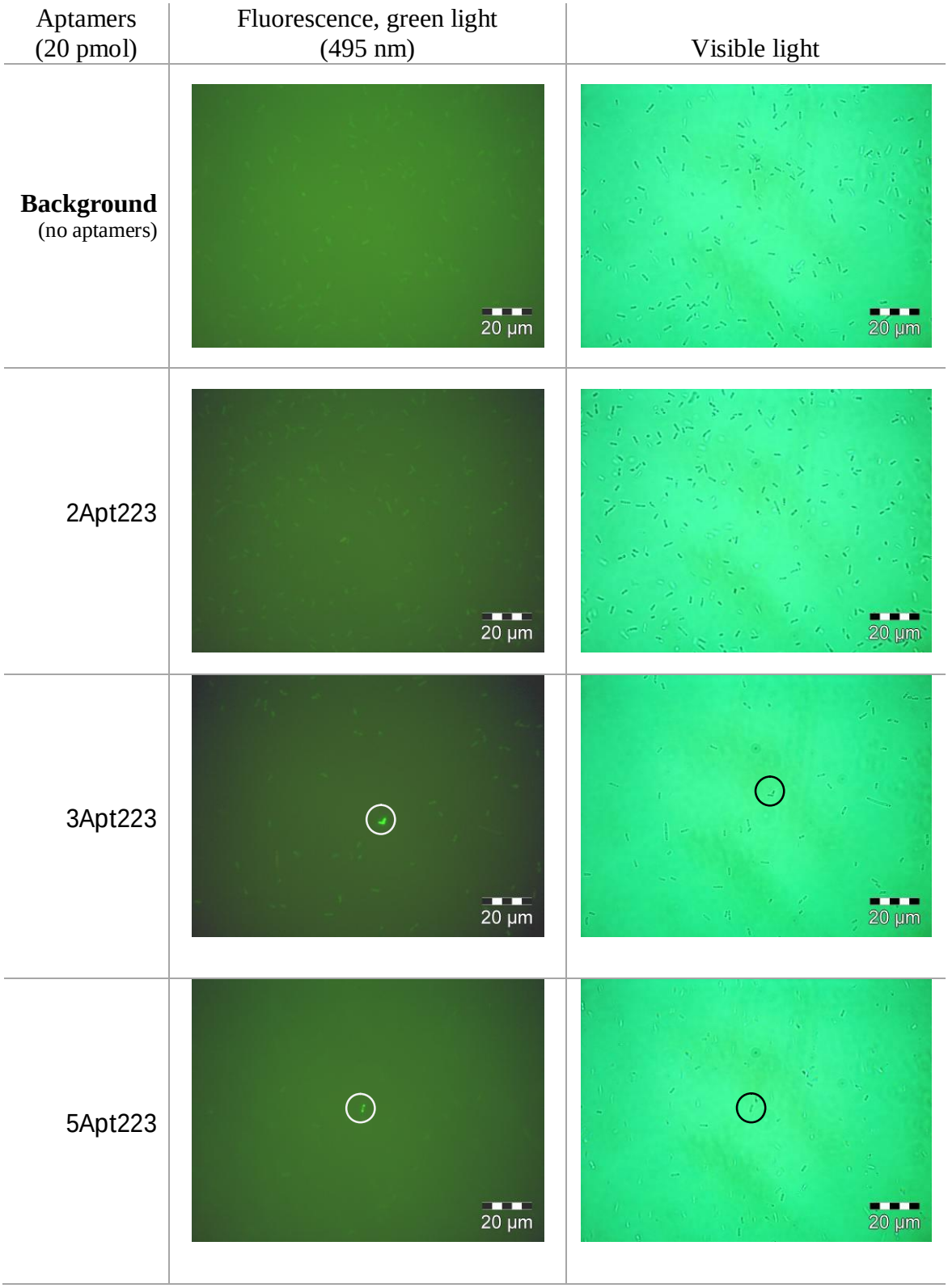


Figure 7.21 Microscopy images of FAM-labelled aptamers 2Apt223, 3Apt223 and 5Apt223 (20 pmol) binding to the surface of *S. typhimurium* 223. Images were taken with a fluorescence microscope with 100× magnification with a green (495 nm) and visible light. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

7.3.5.4. Specificity of the *S. typhimurium* binding aptamers

The specificity of the *S. typhimurium* aptamers 2Apt223, 3Apt223 and 5Apt223 was tested. The aptamers (20 pmol) were incubated with *E. coli* K12, *L. plantarum* and *S. enteritidis* and *S. typhimurium*. The binding was then analysed by fluorimetry and the fluorescence values are shown in Figure 7.22. It can be seen in the figure that the fluorescence is significantly higher in samples 3AptK12 (F=17.3, P=2.3×10^{-3}) and 5AptK12 (F=4.59, P=0.04) when incubated with *S. typhimurium* compared to when they were incubated with *E coli* K12, *L. plantarum* and *S. enteritidis*. The fluorescence of the aptamer 2Apt223 is not significantly higher when incubated with *S. typhimurium* (F=2.93, P=0.1) compared to the values from *E coli* K12, *L. plantarum* and *S. enteritidis* samples. The lower detection for this aptamer was also seen in Figure 7.20. The aptamer 3Apt223 seem to be the strongest of these three aptamers according to the fluorescence values. This was expected because similar result was seen in the aptamer binding study in Figure 7.20 when 20 pmol aptamers was used. These results indicate the aptamer 3Apt223 is having the strongest affinity and specificity of these three aptamers against *S. typhimurium*. It is possible that more specific aptamers could have been obtained by performing the counter selection.

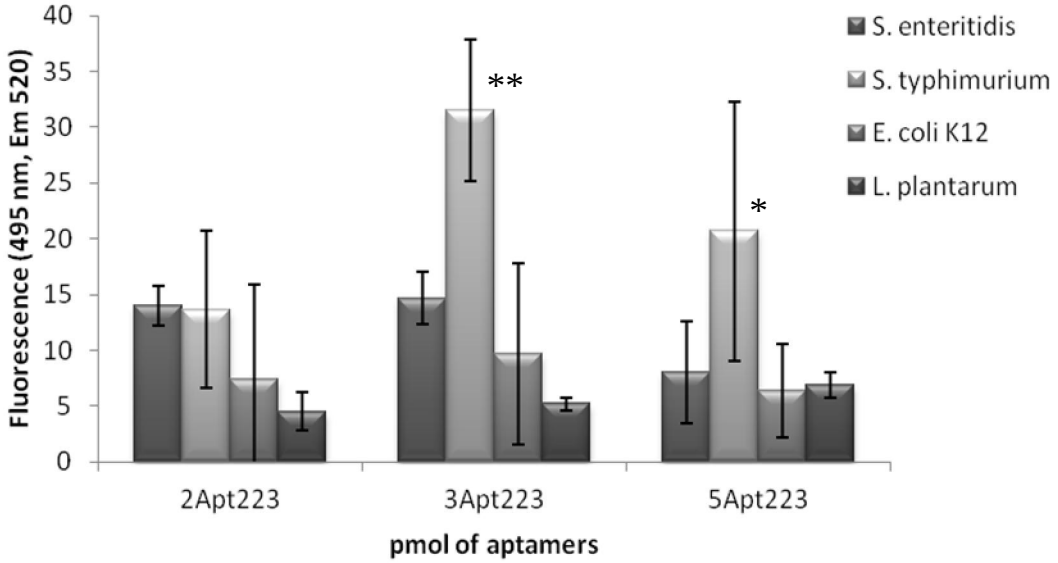


Figure 7.22 Specificity of *S. typhimurium* 223 aptamers. Fluorescence (495 nm, Em 520) was measured by a plate reader in triplicate (n=3). The values are presented as means ± s.d. Fluorescence has been corrected for background (*E. coli* K12=0; other bacteria=0). ** highly significant (P<0,01), *= significant (p≤0,05).

The samples were visualised under the microscope. As an example the microscope images for *S. typhimurium, S. enteritidis, E. coli* K12 and *L. plantarum* samples with 3Apt223 aptamers are presented in Figure 7.23 and Figure 7.24. The fluorescence light images are presented on the left hand side and the visible images on the right hand side. Samples 2Apt223 and 5Apt223 are not presented here because there is no great difference between the samples. A bright spot can be seen in the *S. typhimurium* fluorescence image (left hand side Figure 7.23) when the aptamers have been added. In *S. enteritidis* and *L. plantarum* fluorescence images (left hand side, Figure 7.23 and Figure 7.24) no such a bright spots can be detected when the aptamers were added. In *E. coli* K12 sample (Figure 7.24) the background control has some brighter spots. It was noticed that when this bacterium was looked at under the 100× magnification the background samples express some auto-fluorescence. These green dots are most likely the nucleus inside the bacterial cells. The auto-fluorescence was not visible when the bacterial cells were looked at under the 60× magnification (Figure 4.11). In the *E. coli* K12 background sample bright green dots can be detected. When the aptamers were added, the background fluorescence can be seen in the images but also some brighter spots. This results show that some binding of the *S. typhimurium* aptamers can be detected against *E. coli* K12.

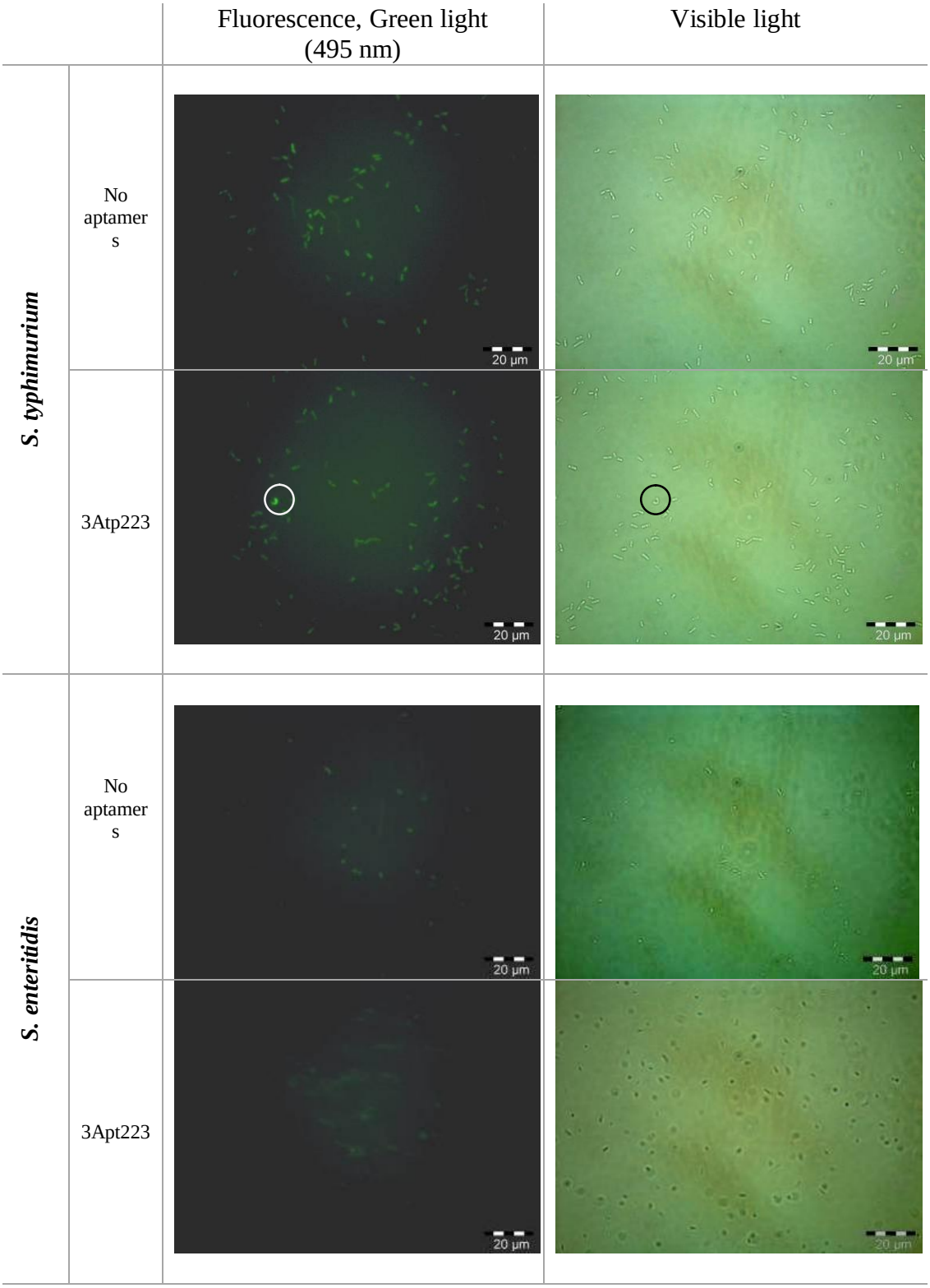


Figure 7.23 Microscopy images showing the binding of the FAM-labelled aptamer 3Apt223 to *S. typhimurium* and *S. enteritidis*. Images were taken with a fluorescence microscope with 100× magnification with a green (495 nm) and visible light. Circles indicate example bacterial cells that can be seen in both, fluorescent and visible light images.

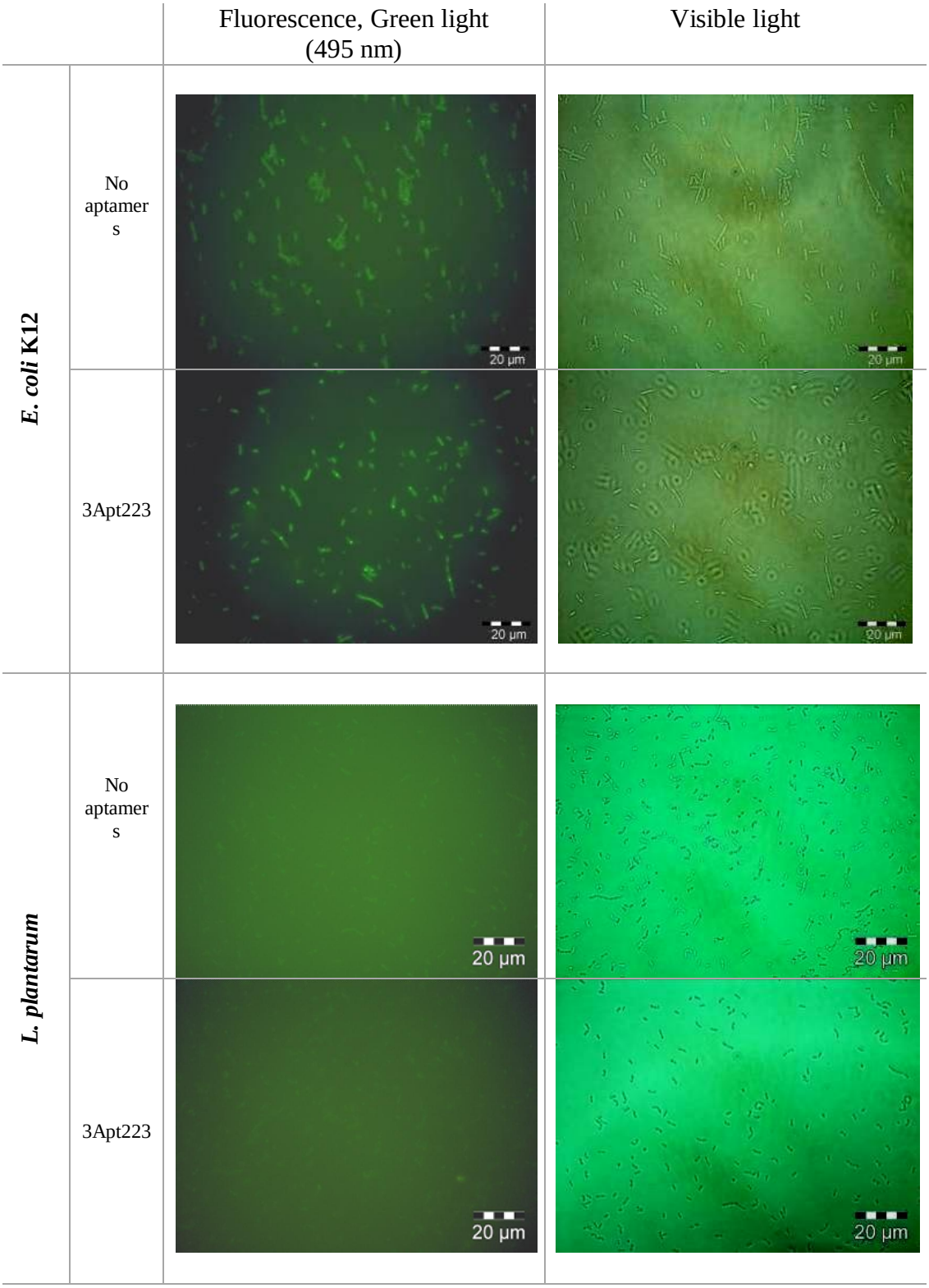


Figure 7.24 Microscopy images showing the binding of the FAM-labelled aptamer 3Apt223 to *E. coli* K12 and *L. plantarum*. Images were taken with a fluorescence microscope with 100× magnification with a green (495 nm) and visible light.

7.4. CONCLUSIONS

Aptamers were selected to bind to pathogenic *E. coli* 496, *E. coli* O157 497, *L. innocua* 17, *L. monocytogenes* 489, *L. monocytogenes* 490, *S. enteritidis* 1152 and *S. typhimurium* 223. The binding of the aptamer pools was tested against their targets. The ninth aptamer pools against *E. coli* O157 497 and *S. typhimurium* 223 were selected for cloning and sequencing. The aptamer structures were then analysed and three sequences for each strain were synthesised with fluorescent labels. These six specific sequences have not previously been reported in scientific literature.

The detection of the aptamer pools binding to bacterial cell surfaces did not show similar results as those reported for the aptamers that were previously selected against *E. coli* K12 (4.3.1.3). The fluorescence values were not as high as for *E. coli* K12. One reason could be that there were not enough aptamer molecules with the right binding properties in the pool. Also the aptamer concentrations were not measured in this experiment, but similar amounts of PCR products were used. The counter selection that was not performed in this study has previously shown to be enriching the aptamer pools (3.3.2). That enrichment was seen as strong bands in the agarose gels when the aptamers were amplified by PCR.

The pathogen strains used in this study were behaving differently than the *E. coli* K12 that forms a good pellet on the bottom of the tube when the samples were centrifuged. Because the pathogenic strains did not always form such a compact pellet, a greater number of bacterial cells might have been washed off during the washing steps.

Two aptamer pools against pathogens (*E. coli* O157 and *S. typhimurium* 223) were selected for cloning and sequencing. The binding of these aptamers was then tested against their targets. The three synthesised sequences against pathogenic *E. coli* O157 were showing some binding and the aptamers against *S. typhimurium* showed a good binding when the fluorimetric analysis was used. Some differences in binding were detected between different aptamers. The binding of the *E. coli* O157 aptamers, that were selected from a pool of *E. coli* K12 aptamers, was tested against *E. coli* K12 bacterial cells. The results showed that these aptamers can also bind to *E. coli* K12 but the binding is not as strong as the binding of the *E. coli* K12 specific

aptamers. The specificity test that was performed to *S. typhimurium* aptamers showed that some of the aptamers were capable of detecting other bacterial cells and only some of the aptamers were specific to *S. typhimurium*.

More aptamers could be selected and identified in order to find molecules with greater specificity. It is possible that more specific sequences with more affinity to their target can be found if a larger number of aptamers are identified and more rounds of selection carried out. It is also possible that more specific aptamers can be found if the original DNA library is larger than used in this study. This would mean that there are more potential binding molecules in the solution and therefore high affinity aptamers against different targets are more likely to be found. The binding properties of the aptamer pools should be controlled after each round of selection to obtain the strongest binding aptamers.

Overall this study showed that the aptamers can be selected to bind to food-borne pathogens using the method developed in this study.

CHAPTER 8 – General discussion and Future work

8.1. GENERAL DISCUSSION

The aim of this study was to develop DNA aptamers with applications in the area of food safety and quality assurance. First, the aptamer selection method based on the commonly used SELEX method was developed following the aptamer selection of the aptamers against common food pathogens. Testing the aptamers' functionality in real food matrices was one of the main objectives of this study. The main steps of work undertaken are presented in Figure 8.1.

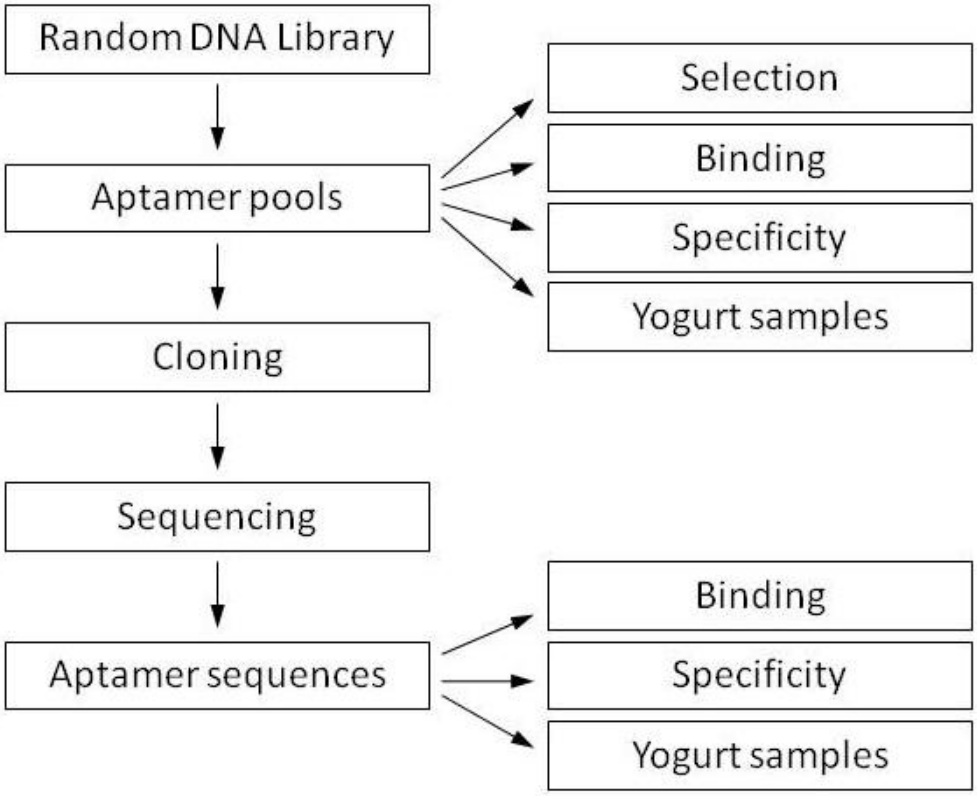


Figure 8.1 An overview of the work completed.

The first objectives were to develop the DNA library and select the aptamers to whole live *E. coli* K12 bacterial cells. There are many things to consider when designing the DNA library, such as shortened products and over amplification (Musheev & Krylov, 2006). The importance of the library design was previously discussed (3.3.1). A 100 nt DNA library was successfully produced (3.3.1) and the aptamers were selected (3.3.2). A filtration based technique (Vivekananda & Kiel,

2006) was used first, but a centrifugation based technique (Chen *et al.,* 2007; Hamula *et al.,* 2008), that was easier to perform, was used to select the aptamers. The aptamers were successfully selected to bind to live non-pathogenic *E. coli* K12 cells by following the protocol of Hamula *et al.* (2008) with some modifications. The binding of the aptamer pools, as well as the individual aptamers can be demonstrated in several different ways. Biotin-labelled (Bruno & Kiel, 1999; Mir *et al.,* 2006; Vivekananda & Kiel, 2006; Murphy *et al.,* 2003; Terazono *et al.,* 2010) and fluorescence-labelled aptamers (Hoffmann *et al.,* 2007; Cao *et al.,* 2009; Joshi *et al.,* 2009) were used here. The binding of the selected aptamers was first demonstrated by an enzymatic detection (3.3.4) that required many washing steps, as previously discussed (3.3.4). A more direct fluorescence based method was then developed (4.3.1) and the aptamers were more easily detected. The bound fluorescent aptamers can be directly detected using fluorescence microscopy (Ohk, *et al.,* 2010) or flow cytometry (Wang *et al.,* 2003; Hoffmann *et al.,* 2007; Cao *et al.,* 2009; Terazono *et al.,* 2010). In this study the binding of the aptamers was successfully shown by fluorescence microscopy and by comparing the fluorescence values measured fluorimetrically with microplate reader. The fluorescence microscopy was a good tool to visualise binding to the bacterial cells whilst fluorimetry was much better as a means of detecting and quantifying aptamer binding.

In order to synthesise the aptamers, the nucleotide sequence of the individual aptamers had to be identified. It is well known that the aptamer sequences can be produced by cloning the aptamer pool by using competent bacterial cells (Ellington & Szostak, 1990). The objective was to clone the aptamers to a plasmid vector, make the copies of the aptamer sequences and then sequence them. The aptamer pool selected to bind *E. coli* K12 was successfully cloned (5.3.1) and individual aptamers were sequenced (5.3.3). The aptamer structures were analysed and four aptamers were produced with the fluorescence labels. The binding properties of these aptamers were then tested with the same fluorimetry technique that was used for aptamer pool detection (5.3.4). All four individual aptamers bound specifically to the target bacteria, although some differences in the binding affinity between each aptamer were seen. These individual aptamer sequences that were specific to *E. coli* K12 have not previously been published in scientific literature.

Aptamers have potential to be used as detection agents for pathogenic micro-organisms in food matrices (Bruno, 2009; Bruno *et al.,* 2009; Joshi *et al.,* 2009). One of the main objectives of this study was to find out if the aptamers selected would stay active when used in a real food matrix. In this study, tap water was first used as a sample matrix to detect live *E. coli* K12 bacterial cells and natural probiotic yogurt was used as a food matrix (6.3.2). The aptamer pool was first used to demonstrate the detection of the bacterial cells from yogurt, followed by a successful detection of the bacteria with the individual aptamers. Aptamers have previously been used to capture the bacterial cells from faecal and chicken rinse samples (Joshi *et al.,* 2009) and it has been shown the aptamers can be used to detect bacteria in different matrices such as beef extract, chicken juice containing visible fat globules and milk (Bruno *et al.,* 2009; Bruno, 2009). This study subscribed the first time the aptamers have been used in direct detection of live bacterial cells in yogurt. These findings indicate aptamers are very promising in the development of new type of detection method for food poisoning bacteria.

A non-pathogenic bacterial strain was used for the development of the aptamer selection process, cloning, detection of the aptamers and the aptamers in a food matrix. Another objective of this study was to select specific aptamers that can be used to detect pathogenic food poisoning bacteria. Aptamers have already been selected against some food-borne pathogens such as *C. jejuni* (Bruno *et al*., 2009; Bruno 2009), *L. monocytogenes* (Ohk *et al.,* 2010), *S. aureus* (Cao *et al*., 2009) and *S. typhimurium* (Joshi *et al.,* 2009). In this study the aptamers were selected to bind to pathogenic *E. coli* 496, *E. coli* O157 497, *L. innocua* 17, *L. monocytogenes* 489, *L. monocytogenes* 490, *S. enteritidis* 1152 and *S. typhimurium* 223 without performing the same counter selection (7.3.1) that was done when selecting aptamers against *E. coli* K12. The binding of the aptamer pools was tested using the previously developed fluorimetric methods. The results were not similar to those reported for the aptamers that were previously selected against *E. coli* K12. The fluorescence values did not reach the same level than the values for the *E. coli* K12 samples. Only the aptamers for two strains, *E. coli* O157 (7.3.1.1) and *S. typhimurium* (7.3.1.3), were cloned (7.3.3) and sequenced (5.3.4). The aptamer structures were analysed and three sequences for each strain were synthesised with fluorescent labels and the binding of these aptamers was examined by fluorimetry (7.3.5). All the aptamers

showed binding to their target although some differences in binding was seen between the individual aptamers. Some cross-reactivity was detected for the aptamers raised against *E. coli* O157 towards *E. coli* K12. These six aptamer sequences have not previously been reported in the scientific literature.

Overall this study indicated that aptamers can be easily selected to specifically bind to live bacterial cells. Unlike the usual aptamer selection process where the aptamers are selected to bind to extracted surface molecules (Bruno *et al.* 2009; Homann & Göringer, 1999; Lorger *et al.,* 2003; Takemura *et al*., 2006; Joshi *et al.,* 2009), in this study, the aptamers were successfully selected to bind to whole bacterial cells. Most importantly this study showed that the aptamers could be used in the detection of food-borne pathogens in contaminated food.

8.2. FUTURE WORK

Many detection systems for pathogenic and spoilage micro-organisms already exist but they are often time consuming (Kärkkäinen *et al.,* 2011b). This limitation is increasingly out of step with the trends observed within the food supply chain that have occurred in response to expanding populations and consumers with decreasing cooking skills. In response to these pressures, there has been a progressive shift towards high volume, centralised food production, combined with rapid distribution of ready meals to retailers. This has served to decrease the time between initial production and consumption and has therefore increased the need for rapid diagnostics to protect consumers from potential exposure to food pathogens. High specificity and affinity single-stranded nucleic acid aptamers have been shown to have a great potential for use in future food safety in detection of bacteria (McMasters & Stratis-Cullum, 2006; Bruno, 2009; Bruno *et al.,* 2009, Joshi *et al*., 2009; Kärkkäinen *et al.,* 2011a).

Aptamers have the potential to be further developed into methods wherein direct detection of pathogens without prior enrichment is needed (Joshi *et al*., 2009) and the pathogens can be directly detected from different food matrices (Bruno *et al*., 2009). More research needs to be done to rapidly select specific aptamers that bind different micro-organisms and to develop aptamer-based assays which can be converted from

the laboratory environment to field. It is already known that the aptamer-based techniques can be used to collect the pathogenic micro-organisms from food matrices (Joshi *et al*., 2009) and the detection of the bacteria could then subsequently be done with using already existing detection methods such as methods based on PCR. This could be one way of using the aptamers but also aptasensor detection (1.3) or direct bacterial detection by aptamers is possible, as shown in this study.

Aptamers could possibly be used as a part of an existing detection system as extensively discussed (1.3). The commonly used biosensor platforms for pathogen detection are antibodies and nucleic acid probes (Torres-Chavolla & Alocilja, 2009). One example, where specific antibodies have been used is a Biacore® detection system based on surface plasmon resonance (Fratamico *et al.,* 1998; Leonard *et al.,* 2004). It has been discussed that the possibility of changing from antibody-based detection systems to using aptamers would also provide several additional ethical and practical benefits such as the elimination of the use of animals and the ability to produce standardised materials suitable for production of test kits or devices by eliminating the inherent biological variability associated with antibodies (Kärkkäinen *et al.,* 2011b). These biosensor systems could be called aptasensors and it has been suggested that they could be used in detection of pathogenic micro-organisms and viruses (Torres-Chavolla & Alocilja, 2009). Aptasensors have created interest because they are easy to handle and they are stable compared to biologically generated proteins. They are also chemically more stable than antibody-affinity biosensors (Liss, *et al*. 2002; Tombelli *et al.* 2005; Liu *et al.* 2010).

To speed up the aptamer selection some alternative selection techniques can be considered as previously discussed (3.3.2). Aptamer structures have also been generated to small molecules such as cocaine in a rational engineering way (Zhang *et al.,* 2008). There are also some automated selection systems that have been developed. Berezovski *et al.* (2006a) described a technique where the aptamers can be selected to bind protein targets with an automated system where no time consuming SELEX is needed. The method used for partitioning is called Non-Equilibrium Capillary Electrophoresis of Equilibrium Mixtures (NECEEM) (Berezovski *et al.* 2006b). This process involves repetitive steps of partitioning with no amplification between them. The technique also allows the clones to be screened

prior to sequencing, so that only clones with suitable binding parameters are sequenced. This automated protocol can be completed in one week. In this method the manual washing steps and the PCR amplification steps are not necessary and the automated system carries on the work normally have to be done by hand. Other automated selection protocol was described by Cox & Ellington (2001) where anti-lysozyme aptamers that function as efficient inhibitors of cell lysis were selected using an automated workstation. This robotic workstation can carry out eight selections in parallel, and will complete approximately 12 rounds of selection in two days. Also Yamamoto & Sen (2010) developed a technique where the aptamers were selected without the normal amplification steps. Compared to the selection method used in this study (2.3.6), the amplification was needed after each round of selection and therefore the selection time is significantly longer. Also the partitioning and the washing steps have to be done manually.

The possibility of introducing a computationally assisted method to study aptamer-protein interaction has been evaluated (Bini *et al.*, 2011). The aim of the study was to streamline screening and selection of new aptamers. A good correlation was found between *in silico* selection and experimental results. The computational method can identify high affinity binding sequences from low affinity sequences reported in literature. The partial pre-selection by using the computational method could provide a significant improvement on the efficiency and effectiveness of aptamer selection and could also speed up the process.

The properties of the aptamers selected in this study could also be further evaluated. For example, evaluation of the aptamer binding molecules on the surface of the bacterial cells might help explain why only some of the bacterial cells within the culture were detected (4.3.1.3). A potential problem with this strategy may be that the molecular structures change during the purification process, and as a result recognition by the aptamers might be lost Hamula *et al.* (2008). It has also been considered that the evaluation of both the binding affinity and specificity of the aptamers is important. One possible way of doing this would be to use methods based on SPR such as the Biacore® system (Näslund *et al.*, 2006). The Biacore® system offers sensor chips that can be used to study the interaction of the molecules without the need for prior labelling. Aptamers could be immobilised on the surface

of the sensor chip in various ways. For example, biotinylated aptamers could be used with a streptavidin coated chip (Hoon *et al.*, 2011) if a specific orientation is required, or alternatively unlabelled aptamers can be bound to chips that have non-specific nucleic acid binding functionality (Bruno *et al*., 2009). This strategy would allow selection of the aptamer with optimal binding performance which is necessary for detecting the low bacterial counts that may be present in contaminated food.

More aptamers could be selected and identified in order to find molecules with a greater specificity. It is possible that more specific sequences with more affinity to their target can be found if a larger number of aptamers is identified. It is also possible that more specific aptamers can be found if the original DNA library is larger than the one used in this study. Enlargement of the library would lead to a more complex DNA library (James, 2000) that contains more molecules which can bind to the target and therefore high affinity aptamers against different targets are more likely to be found. The binding properties of the aptamer pools should be controlled after each round of selection to obtain the strongest binding aptamers. The handling difficulties of the large number of samples became a problem when screening the individual aptamers in this study. All different samples, also the replicates, were produced in separate tubes making the technique difficult to perform. If more samples could be screened at the same time the aptamer screening process would be much faster and that could also improve the aptamer selection. One possible system could be by using a 96-well microplate where more samples can be screened in one go or flow cytometry based method (Wang *et al.,* 2003; Hoffmann *et al.,* 2007; Cao *et al.,* 2009; Terazono *et al.,* 2010) that possibly makes the screening faster.

More detailed investigation of the aptamer motifs would release further information about the aptamer binding properties. More specific aptamers, or even completely new aptamers, could possibly be found by only examining the sequences and the structures of the aptamers (Bini *et al.,* 2011) as previously discussed (7.3.4). It would also be necessary to test the aptamers functionality in different food matrices other than yogurt. Aptamers could be added on the surface of a solid food such as meat or cheese to ascertaining if bacterial contamination could be detected. Aptamers functionality could also be tested in many different food matrices such as jams,

marmalades, jellies, other dairy products (such as milk), meat products, poultry products, fish products and bakery products as listed by Kärkkäinen *et al.* (2011a).

It has been considered that aptamers could be selected to interfere with the intracellular communication between bacterial cells known as quorum sensing (QS) (Kievit de & Iglewski, 2000). Potentially the receptors receiving the growth signals can be blocked by aptamers or the aptamers can be targeted to bind the molecules regulating the signal molecule gene expression leading to the inhibition of bacterial growth. Aptamers could then be used to inhibit the bacterial growth and can possibly be used as an antimicrobial agent. Other possibility would be to incorporate aptamers into the packaging material. Specific aptamers could possible inhibit the bacterial growth or perhaps be used to give a signal if contamination has occurred by such 'smart' packaging (Ozmedir & Floros, 2004).

This study has shown the aptamers can be rapidly selected to specifically detect whole bacterial cells. Aptamers could possibly be used as a part of an existing detection method for pathogens or they can be used for direct detection of the pathogens in food. Very interesting aptamer approaches are aptamer based biosensors where specific aptamer molecules are working as recognition elements and the interaction of the aptamer with the target molecule can be detected in different ways as previously discussed (1.3). The opportunity for large scale synthesis of aptamers combined with their specificity of binding suggests that they have the possibility to inhibit or block the growth of bacteria within food matrices or to prevent binding to host cell surfaces and thus infection. Researchers are actively improving the aptamer based techniques, not only for food pathogen detection, but also in medical and environmental science applications. The future of the aptamer based techniques looks promising.

References

Abubakar, I., Irvine, L., Aldus, C.F., Wyatt, G.M., Fordham, R., Schelenz, S. & Shepstone, L. (2007). A systematic review of the clinical, public health and cost-effectiveness of rapid diagnostic tests for the detection and identification of bacterial intestinal pathogens in faeces and food. *Health Technology Assessment*, **11**, 1–216.

Akitomi, J., Kato, S., Yoshida, Y., Horii, K., Furuichi, M. & Waga, I. (2011). ValFold: Program for the aptamer truncation process. *Bioinformation*, **6,** 38–40.

Armand, P., Kirshenbaum, K., Goldsmith, R.A., Farr-Jones, S., Barron, A.E., Truong, K.T.V., Dill, K.A., Mierke, D.F., Cohen, F.E., Zuckermann, R.N. & Bradley, E.K. (1998). NMR determination of the major solution conformation of a peptoid pentamer with chiral side chains. *Proceedings of the National Academy of Science of the United States of America*, **95**, 4309–4314.

Baeumner, A.J., Cohen, R.N., Miksic, V. & Min, J. (2003). RNA biosensor for the rapid detection of viable Escherichia coli in drinking water. *Biosensors and Bioelectronics*, **18**, 405-413.

Baldrich, E., Restrepo, A. & O'Sullivan, C.K. (2004). Aptasensor development: Elucidation of critical parameters for aptamers performance. *Analytical Chemistry*, **76**, 7053-7063.

Bang, G.S., Cho, S. & Kim, B.G. (2005). A novel electrochemical detection method for aptamer biosensors. *Biosensors and Bioelectronics*, **21**, 863-870.

Berezovski, M., Musheev, M., Drabovich, S. & Krylov, S.N. (2006a). Non-SELEX selection of aptamers. *Journal of the American Chemical Society*, **128**, 1410–1411.

Berezovski, M.V., Musheev, M.U., Drabovich, A.P., Jitkova, J.V. & Krylov, S.N. (2006b). Non-SELEX: selection of aptamers without intermediate amplification of candidate oligonucleotides. *Nature Protocols*, **1**, 1359–1369.

Bertilsson, S., Cavanaugh, C.M. & Polz, M.F. (2002). Sequencing-independent method to generate oligonucleotide probes targeting a variable region in bacterial 16S rRNA by PCR with detachable primers. *Applied and Environmental Microbiology*, **68**, 6077–6086.

Bini, A., Mascini, M., Mascini, M. & Turner, A.P.F. (2011). Selection of thrombin-binding aptamers by using computational approach for aptasensor application. *Biosensors and Bioelectronics*, **26**, 4411–4416.

Bock, L.C., Griffin, L.C., Latham, J.A., Vermaas, E.H. & Toole, J.J. (1992). Selection of single-stranded DNA molecules that bind and inhibit human thrombin. *Nature*, **355**, 564–566.

Bonwick, G.A. & Smith, C.J. (2004). Immunoassays: their history, development and current place in food science and technology. *International Journal of Food Science and Technology*, **39**, 817–827.

Brockstedt, U., Uzarowska, A., Montpetit, A., Pfau, W. & Labuda, D. (2004). In vitro evolution of RNA aptamers recognizing carcinogenic aromatic amines. *Biochemical and Biophysical Research Communications*, **313**, 1004–1008.

Bruno, J. G. (1997). In Vitro selection of DNA to chloroaromatics using magnetic microbead-based affinity separation and fluorescence detection. *Biochemical and Biophysical Research Communications*, **234**, 117–120.

Bruno, J.G. & Kiel, J.L. (1999). In vitro selection of DNA aptamers to anthrax spores with electrochemiluminescence detection. *Biosensors & Bioelectronics*, **14**, 457–464.

Bruno, J.G. (2009). Methods of producing homogeneous plastic-adherent aptamer-magnetic bead-fluorophore and other sandwich assays. Patent, **104**,075 A2.

Bruno, J.G., Phillips, T., Carillo, M.P. & Crowell, R. (2009). Plastic-Adherent DNA aptamer-magnetic bead and quantum dot sandwich assay for Campylobacter detection. *Journal of Fluorescence*, **19**, 427–435.

Cai, H., Lee, T.M-H. & Hsing, I-M. (2006). Label-free protein recognition using an aptamer-based impedance measurement assay. *Sensors and Actuators*, **B 114**, 433–437.

Campbell, M.K. & Farrell, S.O. (2009). Nucleic acid biotechnology techniques. In: Biochemistry, International Students Edition, (6th ed.). 751 p. Thomson Brooks/Cole, Thomson Higher Education, Belmont, CA, USA. Printed in Canada.

Cao, X., Li, S., Chen, L., Ding, H., Xu, H., Huang, Y. Li, J., Liu, N., Cao W., Zhu, Y., Shen, B. & Shao, N. (2009). Combining use of a panel of ssDNA aptamers in the detection of Staphylococcus aureus. *Nucleic Acids Research*, **37,** 4621–4628.

Chen, F., Zhou, J., Luo, F., Mohammed, A-B. & Zhang, X-L. (2007). Aptamer from whole-bacterium SELEX as new therapeutic reagent against virulent Mycobacterium tuberculosis. *Biochemical and Biophysical Research Communications*, **357**, 743–748.

Cheng, A.K.H, Ge, B. & Yu, H.Z. (2007) Aptamer-based biosensors for label free voltammetric detection of lysozyme. *Analytical Chemistry*, **79**, 5158-5164.

Cheng, A.K.H, Sen, D. & Yu, H-Z. (2009). Design and testing of aptamer-based electrochemical biosensors for proteins and small molecules, *Bioelectrochemistry*, **77**, 1-12.

Cox, A., Tilley, A., McOdimba, F., Fyfe, J., Eisler, M., Hide, G. & Welburn, S. (2005). A PCR based assay for detection and identification of African trypanosome species in blood. *Experimental Parasitology*, **111**, 24–29.

Cox, H.C. & Ellington, A.D. (2001), Automated selection of anti-protein aptamers. *Bioorganic & Medicinal Chemistry*, **9**, 2525–2531.

Cummins, L.L., Owens, S.R., Risen, L.M., Lesnik, E. A., Freier, S.M., McGee, D., Guinosso, C.J & Cook, P.D. (1995). Characterization of fully 2'-modified oligoribonucleotide hetero- and homoduplex hybridization and nuclease sensitivity. *Nucleic Acids Research*, **23**, 2019–2024.

De-los-Santos-Alvarez, N., Lobo-Castanon, M.J., Miranda-Ordiers, A.J. & Tunon-Blanco, P. (2008). Aptamers as recognition elements for label-free analytical devices. *Trends in Analytical Chemistry*, **27**, 437-446.

Dennis, D.T., Inglesby, T.V., Henderson, D.A., Barlett, J.G., Ascher, M.S., Eitzen, E., Fine, A.D., Friedlander, A.M., Hauer, J., Layton, M., Lillibridge, S.R., McDade, J.E., Osterholm, M.T., O'Toole, T., Parker, G., Perl, T.M., Russel., P.K. & Tonat, K. (2001). Tularemia as a biological weapon. Medical and public health management. *The Journal of American Medical Association*, **291**, 2769–2773.

Dougan, H., Lyster, D.M., Vo, C.V., Stafford, A., Weitz, J.I. & Hobbs, J.B. (2000). Extending the lifetime of anticoagulant oligodeoxynucleotide aptamers in blood. *Nuclear Medicine & Biology*, **27**, 289–297.

Ellington, A.D. & Szostak J.W. (1990). In vitro selection of RNA molecules that bind specific ligands. *Nature*, **346**, 818–822.

Eurofins MWG Operon. DNA sequencing Result Guide (2011). Available online: www.eurofinsdna.com.

European Food Safety Authority, European Centre for Disease Prevention and Control (2011). The European Union Summary Report on Trends and Sources of Zoonoses, Zoonotic Agents and Food-borne Outbreaks in 2009. EFSA Journal, 2011 9(3):2090 [378 pp.]. Available at http://www.efsa.europa.eu/en/efsajournal/pub/2090.htm.

Evtugyn, G., Porfireva, A., Ryabova, M. & Hianik, T. (2008). Aptasensor for thrombin based on carbon nanotubes-methylene blue composites. *Electroanalysis* **20**, 2310–2316.

Fischer, N.O., Tarasow, T.M. & Tok, J.B-H. (2007). Aptasensors for biosecurity applications. *Current Opinion in Chemical Biology*, **11**, 316–328.

Fratamico, P.M., Strobaugh, T.P., Medina, M.B. & Gehrin, A.G. (1998). Detection of Escherichia coli 0157:H7 using a surface plasmon resonance biosensor. *Biotechnology Techniques*, **12**, 571–576.

Freeman, R., Girsh, J., Jou, A.F., Ho, J.A., Hug, T., Dernedde, J. & Willner, I. (2012). Optical aptasensors for the analysis of the vascular endothelial growth factro (VEGF). *Analytical Chemistry*, **84**, 6192-9198.

Geiger, A., Burgstaller, P., von der Eltz, H., Roeder, A. & Famulok, M. (1996). RNA aptamers that bind L-arginine with sub-micromolar dissociation constants and high enantioselectivity. *Nucleic Acids Research*, **24**, 1029–1036.

Gening, L.V., Klincheva, S.A., Gusev, A.S., Surovoy, A.Y. & Potapov, V.K. (2001). SSCP screening of individual aptamers, *BioTechniques*, **31**, 828–834.

González, M., Bagatolli, L.A., Echabe, I., Arrondo, J.L.R., Argaraña, C.E., Cantor, C.R. & Fidelio, G.D. (1997). Interaction of biotin with streptavidin, Thermostability and conformational changes upon binding. *The Journal of The Biological Chemistry*, **272**, 11288–11294.

Gopinath, S.C.B., Misono, T.S., Kawasaki, K., Mizuno, T., Imai, M., Odagiri, T. & Kumar, P.K.R. (2006). An RNA aptamer that distinguishes between closely related human influenza viruses and inhibits haemagglutinin-mediated membrane fusion. *Journal of General Virology*, **87**, 479–487.

Green, L. S., Jellinek, D., Jenison, R., Östman, A., Heldin, C-H. & Janjic, J. (1996). Inhibitory DNA ligands to Platelet-Derived Growth Factor B-Chain. *Biochemistry*, **35**, 14413–14424.

Hall, B., Cater, S., Levy, M. & Ellington, A.D. (2009). Kinetic optimization of protein-responsive aptamer beacon. *Biotechnology and Bioengineering*, **103**, 1049-1059.

Hamula, C.L.A., Guthrie, J.W., Zhang, H., Li, X-F. & Le, X.C. (2006). Selection and analytical applications of aptamers. *Trends in Analytical Chemistry*, **25**, 681–691.

Hamula, C.L.A., Zhang, H., Guan, L.L., Li, X-F. & Le, X.C. (2008). Selection of aptamers against live bacterial cells. *Analytical Chemistry*, **80**, 7812–7819.

Herne, T.M. & Tarlov, M.J. (1997). Characterization of DNA probes immobilized on gold surfaces. *Journal of American Chemistry Sociaty*, **119**, 8916-8920.

Hesselberth, J.R., Miller, D., Robertus, J. & Ellington, A.D. (2000). In vitro selection of RNA molecules that inhibit the activity of ricin A-chain. *The Journal of Biological Chemistry*, **275,** 4937–4942.

Hianik, T., Ostatna, V., Sonlajtnerova, M. & Grman, I. (2007). Influence of ionic strength, pH and aptamer configuration for binding affinity to thrombin. *Bioelectrochemistry*, **70**, 127-133.

Hicke, B.J., Watson, S.R., Koenig, A., Lynott, C.K., Bargatze, R.F., Chang, Y-F., Ringquist, S., Moon-McDermott, L., Jennings, S., Fitzwater, T., Han, H-L., Varki, N., Albinana, I., Willis, M.C., Varki, A. & Parma, D. (1996). DNA aptamers block L-selectin function in vivo. Inhibition of human lymphocyte trafficking in SCID mice. *The Journal of Clinical Investigation*, **98**, 2688–2692.

Ho, M.Y., D'Souza, N. & Migliorato, P. (2012). Electrochemical aptamer-based sandwich assay for the detection of explosives. *Analytical Chemistry*, **84**, 4245-4247.

Hoffmann, J., Paul, A., Harwardt, M., Groll, J., Reeswinkel, T., Klee,D., Moeller, M., Fischer, H., Walker, T., Greiner, T., Ziemer, G. & Wendel, H.P. (2007). Immobilized DNA aptamers used as potent attractors for porcine endothelial precursor cells. *Journal of Biomedical Materials Research* Part A, **84**A, 614–621.

Homann, M. & Göringer, U. (1999). Combinatorial selection of high affinity RNA ligands to live African trypanosomes. *Nucleic Acids Research*, **27**, 2006–2014.

Hoon, S., Zhou, B., Janda, K.D., Brenner, S. & Scolnick, J. (2011). Aptamer selection by high-throughput sequencing and informatics analysis. *Biotechniques*, **51**, 413–416.

Hughes, S. (Ed.), & Moody, A. (Ed.). (2007). The Methods Express series: PCR. Scion Publishing Limited, Bloxham, UK.

Huizenga, D. & Szostak, J.W. (1995). A DNA aptamer that binds adenosine and ATP. *Biochemistry*, **34,** 656–665.

Hunt, D.E., Klepac-Ceraj, V., Acinas, S.G., Gautier, C., Bertilsson, S. & Polz, M.F. (2006). Evaluation of 23S fRNA PCR primers for use in phylogenetic studies of bacterial diversity. *Applied and Environmental Microbiology*, **72**, 2221–2225.

Invitrogen. TOPO TA Clonin® Kit for Sequencing. (2006). Users Manual. Version O.

Iqbal, S.S., Mayo, M.W., Bruno, J.G., Bronk, B.V., Batt, C.A. & Chambers, J.P. (2000). A review of molecular recognition technologies for detection of biological threat agents. *Biosensors & Bioelectronics*, **15**, 549–578.

James, W. (2000). Aptamers. In: Encyclopedia of Analytical Chemistry, (Edited by R.A. Meyers). Available on Wiley Online Library (http://onlinelibrary.wiley.com).

James, W. (2007). Aptamers in the virologists' toolkit. *Journal of General Virology*, **88**, 351–364.

Jay, J.M. (2000). Modern Food Microbiology, (6th ed.). 679 p. Aspen Publishers, Inc. Gaithesburg, Maryland.

Joshi, R., Janagama, H., Dwivedi, H.P., Senthil Kumar, T.M.A., Jaykus, L-A., Schefers, J. & Sreevatsan, S. (2009). Selection, characterization, and application of DNA aptamers for the capture and detection of Salmonella enterica serovars. *Molecular and Cellular Probes*, **23**, 20–28.

Kang, J., Lee, M.S. & Gorenstein, D.G. (2005). The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: Application to in vitro combinatorial selection of aptamers, *Journal of Biochemical and Biophysical Methods*, **64,** 147–151.

Karoonuthaisiri, N., Charlermroj, R., Uawisetwathana, U., Luxananil, P., Kirtikara, K. & Gajanandana, O. (2009). Development of antibody array for simultaneous detection of foodborne pathogens. *Biosensors & Bioelectronics*, **24,** 1641–1648.

Kawakami, J., Imanaka, H., Yokota, Y. & Sugimoto, N. (2000). In vitro selection of aptamers that act with Zn2+. *Journal of Inorganic Biochemistry*, **82**, 197–206.

Kievit, de, T.R. & Iglewski. B.H. (2000). Bacterial Quorum sensing in pathogenic relationships. *Infection and Immunity*, **68,** 4839–4849.

Kim, S-E., Su, W., Cho, M., Lee, Y. & Choe, W-S. (2012).Harnessing aptamers for electrochemical detection of endotoxin. *Analytical Biochemistry*, **424**, 12–20.

Kittigul, L., Suthachana, S., Kittigul, C. & Pengruangrojanachai, V. (1998). Immunoglobulin M–capture biotin-streptavidin enzyme-linked immunosorbent assay for detection of antibodies to dengue viruses. *The American Journal of Tropical Medicine and Hygiene*, **59**, 352–356.

Koopmans, M. & Duizer, E. (2004). Foodborne viruses: an emerging problem. *International Journal of Food Microbiology***, 90**, 23–41.

Kuboki, N., Inoue, N., Sakurai, T., Di Cello, F., Grab, D.J., Suzuki, H., Sugimoto, C. & Igarashi, I. (2003). Loop-mediated isothermal amplification for detection of African Trypanosomes. *Journal of Clinical Microbiology*, **41**, 5517–5524.

Kärkkäinen, R.M., Ryun Drasbek, M., McDowall, I., Smith, C.J., Young, N.W.G. & Bonwick, G.A. (2011a). Aptamers. Patent application US 61/553,501.

Kärkkäinen, R.M., Ryun Drasbek, M., McDowall, I., Smith, C.J., Young, N.W.G. & Bonwick, G.A. (2011b). Aptamers for safety and quality assurance in the food industry: detection of pathogens. *International Journal of Food Science and Technology,* **46,** 445–454.

Lai, R. Y., Plaxco, K.W. & Heeger, A.J. (2007). Aptamer-based electrochemical detection of picomolar platelet-derived growth factor directly in blood serum. *Analytical Chemistry,* **79,** 229–233.

Lautner, G., Balogh, Z., Bardoczy, V., Meszaros, T. & Gyurcsanyi, R.E. (2010). Aptamer based biochips for label-free detection of plant virus coat proteins by SPR imaging, *The Analyst,* **135**, 918-926.

Lazcka, O., Del Campo, F.J. & Munõz, F.X. (2007). Pathogen detection: A perspective of traditional methods and biosensors. *Biosensors & Bioelectronics,* **22,** 1205–1217.

Lee M. & Walt, D.R. (2000). A fiber-optic microarray biosensor using aptamers as receptors. *Analytical Biochemistry*, **282**, 142-146.

Lee, J-O., So, H-M., Jeon, E-K., Chang, H., Won, K & Kim, Y.H. (2008). Aptamers as molecular recognition elements for electrical nanobiosensors. *Analytical and Bioanalytical Chemistry*, **390**, 1023-1032.

Lee, S., Kim, Y.S., Jo, M., Jin, M., Lee, D. & Kim, S. (2007). Chip-based detection of hepatitis C virus using RNA aptamers that specifically bind to HCV core antigen. *Biochemical and Biophysical Research Communications*, **358**, 47–52.

Lee, S.W., Gallardo, H.F., Gaspar, O., Smith, C. & Gilboa, E. (1995). Inhibition of HIV-1 in CEM cells by a potent TAR decoy. *Gene Therapy*, **2**, 377–384.

Leonard, P., Hearty, S., Brennan, J., Dunne, L., Quinn, J., Chakraborty, T. & O'Kennedy, R. (2003). Advances in biosensors for detection of pathogens in food and water. *Enzyme and Microbial Technology,* **32**, 3–13.

Leonard, P., Hearty, S., Quinn, J. & O'Kennedy, R. (2004). A generic approach for the detection of whole Listeria monocytogenes cells in contaminated samples using surface plasmon resonance. *Biosensors and Bioelectronics*, **19**, 1331–1335.

Liss, M., Petersen, B., Wolf, H. Prohaska, E. (2002). An aptamer-based quartz crystal protein biosensor, *Analytical Chemistry,* **74**, 4488-4495.

Liu, J. & Lu, Y. (2006). Fast colorimetric sensing of adenosine and cocaine based on a general sensor design involving aptamers and nanoparticles. *Angewandte Chemie International Edition*, **45**, 90-94.

Liu, Y., Tuleouva, N., Ramanculov, E. & Revzin, A. (2010). Aptamer-based electrochemical biosensor for interferon gamma detection. *Analytical Chemistry*, **82,** 8131-8136.

Liu, Z., Yuan, R., Chai, Y., Zhuo, Y., Hong, C., Yang, X., Su, H. & Qian, X. (2009). Highly sensitive, reusable electrochemical aptasensor for adenosine. *Electrochimica Acta*, **54**, 6207–6211.

Lorger, M., Engstler, M., Homann, M. & Göringer, U. (2003). Targeting the variable surface of African Trypanosomes with variant surface glycoprotein-specific, serum-stable RNA aptamers. *Eucaryotic Cell*, **2**, 84–94.

Macaya, R. F., Schultze, P., Smith, F.W., Roe, J.A. & Feigon, J. (1993). Thrombin-binding DNA aptamer forms a unimolecular quadruplex structure in solution. *Proceedings of National Academy of Science of the United States of America* **90**, 3745–3749.

Mandal, M., Lee, M., Barrick, J.E., Weinberg, Z., Emilsson, G.M., Ruzzo, W.L. & Breaker, R.R. (2004). A glycine-dependent riboswitch that uses cooperative binding to control gene expression. *Science*, **306**, 275–279.

McBride, L.J. & Caruthers, M.H. (1983). An investigation of several deoxynucleoside phosphoramidites useful for synthesizing deoxyoligonucleotides. *Tetrahedron Letters*, **24**, 245–248.

McCauley, T.G., Hamaguci, N. & Stanton, M. (2003). Aptamer-based biosensor arrays for detection and quantification of biological macromolecules. *Analytical Biochemsitry*, **319**, 244-250.

McMasters, S. & Stratis-Cullum, D.N. (2006). Evaluation of aptamers as molecular recognition elements for pathogens using capillary electrophoretic analysis. *Proceedings of SPIE*, **6380**, 63800B.

Miller, S.E., Taillon-Miller, P. & Kwok, P-Y. (1999). Cost-Effective staining of DNA with SYBR® Green in preparative agarose gel electrophoresis. *BioTechniques*, **27**, 34–36.

Min, K., Cho, M., Han, S-Y., Shim, Y-B., Ku, J. & Ban, C. (2008). Simple and direct electrochemical detection of interferon using its RNA and DNA aptamers. *Biosensors and Bioelectronics*, **23**, 1819-1824.

Minunni, M., Tombelli, S., Gullotto, A., Luzi, E. & Mascini, M. (2004). Development of biosensors with aptamers as bio-recognition element: the case of HIV-1 Tat protein. *Biosensors and Bioelectronics*, **20**, 1149-1156.

Mir, M., Katakis, I. & Vreeke, M. (2007). Aptamer biosensors: an alternative to immunosensors. *IVD Technology*, May, 39.

Mir, M., Vreeke, M. & Katakis, I. (2006). Different strategies to develop an electrochemical thrombin aptasensor. *Electrochemistry Communications,* **8,** 505–511.

Muhammad-Tahir, Z. & Alocilja, E.C. (2003). A conductometric biosensor for biosecurity. *Biosensors and Bioelectronics,* **18,** 813-819.

Murphy, M.B., Fuller, S.T., Richardson, P.M. & Doyle, S.A. (2003). An improved method for the in vitro evolution of aptamers and applications in protein detection and purification. *Nucleic Acids Research,* **31**, 110–118.

Musheev, M.U., & Krylov, S.N. (2006). Selection of aptamer by systematic evolution of ligands by exponential enrichment: Addressing the polymerase chain reaction issue. *Analytica Chimica Acta*, **564**, 91–96.

Ng, E.W.M., Shima, D.T., Calias, P., Cunningham, Jr., E.T., Guyer, D.R. & Adamis, A.P. (2006). Pegaptanib, a targeted anti-VEGF aptamer for ocular vascular disease. *Nature Reviews - Drug Discovery*, **5**, 123–132.

Nitsche, A., Kurth, A., Dunkhorst, A., Pänke, O., Sielaff, H., Junge, W., Muth, D., Scheller, F., Stöcklein, W., Dahmen, C., Pauli, G. & Kage, A. (2007). One-step selection of Vaccinia virus-binding DNA aptamers by MonoLEX. *BMC Biotechnology,* **7**, 48–60.

Nudler, E. & Mironov, A.S. (2004). The riboswitch control of bacterial metabolism. *TRENDS in Biochemical Sciences,* **29**, 11–17.

Nutiu, R. & Li, Y. (2003). Structure-switching signaling aptamers. *Journal of American Chemical Society,* **125**, 4771-4778.

Nutiu, R. & Li, Y. (2004). Structure-switching signaling aptamers: Tranducing molecular recognition into fluorescence signaling. Chemistry - *A European Journal*, **10**, 1868-1876.

Nutiu, R. & Li, Y. (2005). Aptamers with fluorescence-signaling properties. *Methods*, **37**, 16-25.

Nutiu, R. & Li, Y. (2005). Aptamers with fluorescence-signaling properties. *Methods*, **37,** 16–25.

Näslund, A., Björkelund, H., Stenberg, J., Andersson, K. & Franklin, G. (2006). Rapid, reproducible screening of drug compound–target protein interactions using Biacore A100. *Nature Methods Application Notes,* 14–16.

Ohk, S.H., Koo, O.K., Sen, T., Yamamoto, C.M. & Bhunia, A.K. (2010). Antibody–aptamer functionalized fibre-optic biosensor for specific detection of Listeria monocytogenes from food. *Journal of Applied Microbiology*, **109**, 808–817.

Ozmedir, M. & Floros, J.D. (2004). Active food packaging technologies. *Clinical Reviews in Food Science and Nutrition*, **44**, 185–193.

Pagano, B., Martino, L., Randazzo, A. & Giancola, C. (2008). Stability and binding properties of a modified thrombin binding aptamer. *Biophysical Journal*, **94**, 562–569.

Pan, Q., Zhang, X-L., Wu, H-Y., He, P-W., Wang, F., Zhang, M-S., Hu, J-M., Xia, B., Wu, J. (2005). Aptamers That Preferentially Bind Type IVB Pili and Inhibit Human Monocytic-Cell Invasion by Salmonella enterica Serovar Typhi. *Antimicrobial Agents and Chemotherapy*, **49**, 4052–4060.

Pendergrast, P.S., Marsh, H.N., Grate, D., Healy J.M. & Stanton, M. (2005). Nucleic acid aptamers for target validation and therapeutic applications. *Journal of Biomolecular Techniques*, **16**, 224–234.

Phizicky, E., Bastiaens, P.I.H., Zhu, H., Snyder, M. & Fields, S. (2003). Protein analysis on a proteomic scale. *Nature*, **422**, 208–215.

Pijper, A. (1946). Methylcellulose and bacterial motility. *Journal of bacteriology*, **53**, 257–269.

Potyrailo, R.A., Conrad, R.C., Ellington, A.D. & Hieftje, G.M. (1998). Adapting selected nucleic acid ligands (Aptamers) to biosensors. *Analytical Chemistry*, **70**, 3419-3425.

Promega Corporation. Technical Manual (2009). pGEM®-T and pGEM®-T Easy vector systems, part# TM042. Available online: www.promega.com

Prusiner, S.B. (1998). Prions. Proceedings of the National Academy of Sciences USA, **95**, 13363–13383.

Radwanska, M., Magez, S., Perry-O'Keefe, H., Stender, H., Coull, J., Sternberg, J.M., Büscher, P. & Hyldig-Nielsen, J.J. (2002). Direct detection and identification of African Trypanosomes by fluorescence in situ hybridization with peptide nucleic acid probes. *Journal of Clinical Microbiology*, **40**, 4295–4297.

Sassolas, A., Blum, L.J. & Leca-Bouvier. B.D. (2009) Electrochemical aptasensors. *Electroanalysis*, **21**, 1237-1250.

Song, S., Wang, L., Li, J., Zhao, J. & Fan, C. (2008). Aptamer-based biosensors. *Trends in Analytical Chemistry*, **27**, 108-117.

Steel, A.B., Herne, T.M. & Tarlov, M.J. (1998). Electrochemical quantitation of DNA immobilized on gold. *Analytical Chamistry*, **70**, 4670-4677.

Steinhauer, C., Wingren, C., Hager, A.C. & Borrebaeck, C.A. (2002). Single framework recombinant antibody fragments designed for protein chip applications. *Biotechniques*, Dec Suppl, 38–45.

Stevens, K.A. & Jaykus, L-A. (2004). Bacterial separation and concentration from complex sample matrices: A review. *Critical Reviews in Microbiology,* **30**, 7–24.

Stoltenburg, R., Reinemann, C. & Strehlitz, B. (2007). SELEX—A (r)evolutionary method to generate high-affinity nucleic acid ligands. *Biomolecular Engineering*, **24**, 381–403.

Symensma, T.L., Giver, L., Zapp, M., Takle, G.B. & Ellington, A.D. (1996). RNA aptamers selected to bind Human Immunodeficiency Virus Type 1 Rev in vitro are Rev responsive in vivo. *Journal of Virology,* **70**, 179–187.

Takemura, K.,Wang, P.,Vorberg, I., Surewicz, W., Priola, S.A., Kanthasamy, A., Pottahil, R., Chen, S.G., Sreevatsan, S. (2006). DNA Aptamers that bind to PrPC and not PrPSc show sequence and structure specificity, *Experimental Biology and Medicine,* **231**, 204–214.

Terazono, T., Anzai, Y., Soloviev, M. and Yasuda, K. (2010). Labelling of live cells using fluorescent aptamers: binding reversal with DNA nucleases. J*ournal of Nanobiotechnology*, **8**, 8.

Tombelli, S., Minunni, M. & Mascini M, (2005). Analytical applications of aptamers. *Biosensors and Bioelectronics*, **20**, 2424–2434.

Tombelli, S., Minunni, M. & Mascini, M. (2007). Aptamers-based assays for diagnostics, environmental and food analysis. *Biomolecular Engineering*, **24**, 191–200.

Tombelli, S., Minunni, M., Luzi, E. & Mascini, M. (2005). Aptamer-based biosensors for the detection of HIV-1 Tat protein. *Bioelectrochemistry,* **67**, 135-141.

Torres-Chavolla, E. & Alocilja, E.C. (2009). Aptasensors for detection of microbial and viral pathogens. *Biosensors and Bioelectronics*, **24**, 3175–3182.

Tuerk, C. & Gold, L. (1990). Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science*, **249**, 505–510.

Tuleuova, N., Jones, C.N., Yan, J., Ramanculov, E., Yokobayashi, Y. & Revzin, A. (2010). Development of an aptamer beacon for detection of interferon-gamma. *Analytical Chemistry,* **82**, 1851-1857.

Tyagi, S. & Kramer, R. (1996). Molecular beacons: Probes that fluoresce upon hybridization. *Nature Biotechnology,* **14**, 303-308.

Wang, C., Zhang, M., Yang, G., Zhang, D., Ding, H., Wang, H., Fan, M., Shen, B. & Shao, N. (2003). Single-stranded DNA aptamers that bind differentiated but not parental cells: subtractive systematic evolution of ligands by exponential enrichment. *Journal of Biotechnology*, **102**, 15–22.

Wang, J. (2008). Electrochemical glucose biosensors. *Chemical Reviews*, **108**, 814-825.

Wang, J., Wang, L., Liu, X., Liang, Z., Song, S., Li, W., Li, G. & Fan, C. (2007a). A gold nanoparticle-based aptamer target binding readout for ATP assay. *Advanced Materials*, **19**, 3943–3946.

Wang, Z., Wilkop, T., Xu, D., Dong, Y., Ma, G. & Cheng, Q. (2007b). Surface plasmon resonance imaging for affinity analysis of aptamer–protein interactions with PDMS microfluidic chips. *Analytical and Bioanalytical Chemistry*, **389**, 819-825.

Willner, I. & Zayats, M. (2007). Electronic aptamer-based sensors. *Angewandte Chemie International Edition*, **46**, 6408 – 6418.

Winkler, W., Nahvi, A. & Breaker, R.R. (2002). Thiamine derivatives bind messenger RNAs directly to regulate bacterial gene expression. *Nature*, **419**, 952–956.

Viswanathan, V.K., Krcmarik, K. & Cianciotto, P. (1999). Template secondary structure promotes polymerase jumping during PCR amplification, *Biotechniques*, **27**, 508–511.

Vivekananda, J. & Kiel, J.L. (2003). Methods and compositions for aptamers against anthrax. Patent US 6,5696,30 B1.

Vivekananda, J. & Kiel, J.L. (2006). Anti-Francisella tularensis DNA aptamers detect tularemia antigen from different subspecies by Aptamer-Linked Immobilized Sorbent Assay. *Laboratory Investigation*, **86**, 610–618.

Xu, D.K., Xu, D.W., Yu, X.B., Liu, Z.H., He, W. & Ma, Z.Q. (2005). Label-free electrochemical detection for aptamer-based array electrodes. *Analytical Chemistry*, **77**, 5107-5113.

Yamamoto, C. & Sen, T. (2010). Aptamers that bind to Listeria surface proteins. Patent, US 7,645,582 B2.

Yamamoto, R., Katahira, M., Nishikawa, S. Baba, T., Taira, K. & Kumar, PKR. (2000). A novel RNA motif that binds efficiently and specifically to the Tat protein of HIV and inhibits the trans-activation by Tat of transcription in vitro, *Genes Cells*, **5**, 371-388.

Zelada-Guillen, G.A., Riu, J., Duzgun, A. & Xavier Ruis, F. (2009). Immediate detection of living bacteria at ultralow concentrations using a carbon nanotube based potentiometric aptasensor. *Angewandte chemie international edition*, **48**, 7334-7337.

Zhang, J., Wang, L., Pan, D., Song, S., Boey, F.Y.C., Zhang, H. & Fan, C. (2008). Visual cocaine detection with gold nanoparticles and rationally engineered aptamer structures. *Small*, **4**, 1196–1200.